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"Evolutionary Species" and the Systematics of the Artiodactyla of China in the 21st Century

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Who is innocent in authorship misconduct?

Recently, the editorial office received a requisition from one of the co-corresponding authors of an article published in *Zoological Research* in 2003. This researcher claimed he was not informed that he was listed as an author during the entire manuscript submission and publication process. Moreover, he had a concern about the reliability of the data in the paper. Therefore, he would like to withdraw his authorship of this particular article or withdraw this article entirely. The editorial office forwarded the letter to the other authors to collect comments, and the first author completely denied the co-corresponding author's claim of unawareness of authorship by providing archived emails between them. Setting aside what really happened 13 years ago, in this case, it may be interpreted that either this cocorresponding author himself is announcing his honorary authorship (either by passively being added to the byline or actively accepting the offer) in this article, or is trying to avoid taking (potential) responsibility regarding the research content by using honorary authorship as a defense. Meanwhile, the first author has been accused of offering honorary authorship to a senior researcher.

In scientific writing, the debate regarding authorship, including the definition, eligibility, and order of authorship or contributor, as well as honorary authorship (also called guest or gift authorship) and ghost authorship never stops (Kornhaber et al., 2015; Stretton, 2014; Vinther & Rosenberg, 2012), especially as multidisciplinary cooperation has become increasingly common in almost every aspect of scientific study and in the rush for publication glory. To help researchers define authorship, guidelines written by international organizations are available and are continuously undergoing amendment. For example, of the most commonly accepted authorship measures, the International Committee of Medical Journal Editors (ICMJE) states that an author needs to meet all four of the following criteria: (1) substantial contributions to: the conception or design of the work; or the acquisition, analysis, or interpretation of data; (2) drafting or critically revising the work for important intellectual content; (3) final approval of the version to be published; (4) agreement to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the research are appropriately investigated and resolved (ICMJE, 2013). Contributors who meet fewer than all four of the above criteria should not be listed as authors, but should be acknowledged. It also states that "examples of activities that alone (without other contributions) do not qualify a contributor for authorship are acquisition of funding; general supervision of a research group or general administrative support; and writing assistance, technical editing, language editing, and proofreading" (ICMJE, 2013).

In addition to guidelines such as those mentioned above, many journals also ask each author listed in the byline or acknowledgements to provide a statement that entails the specific contributions and roles every person played. These measures seem comprehensive and should prevent authorial misconduct; however, the actual situation is not as transparent as it should be. Nowadays, the quality of a researcher's publications has become critical in performance evaluation, funding application, and career promotion, to name a few. It is not rare for junior or lower ranked researchers to offer guest authorships to senior researchers or heads of department to either increase the chance of submission acceptance or to obtain certain benefits in resource allocation (Du & Tang, 2013; Kressel & Dixon, 2011). Some researchers may even exchange authorships to multiply publications.

Many anonymous surveys have been conducted regarding unethical or breached authorship, and it is not surprising that authorship violation is a global phenomenon, found within a large number of publications, no matter how high the impact factor nor what language they are published in (Wislar et al., 2011). One reason authorship misconduct is hard to eliminate is that such behaviors are extremely difficult to define. When someone's name is listed in the byline for certain reasons, what good are authorship guidelines, statements or questionnaires? For example, in some academic publication withdrawals, such as the large-scale article withdrawal of Biomed Central articles in 2014 (http://retractionwatch.com), some authors immediately claimed honorary authorship. Nevertheless, if these articles were not involved in the scandal, would those authors continue to enjoy the "honor" from honorary authorship? In regards to the particular article I mentioned at the beginning concerning disputed authorship, who is innocent and who is the victim, I'm afraid I cannot tell.

When a young researcher begins his/her scientific training, the first thing to learn is academic ethics and the fundamentals of scientific spirit. Proper authorship embodies honesty, integrity, fairness and transparency, which surely are the very essence of any scientific pursuit. To avoid authorial misconduct, besides explicit instructions, detailed questionnaires, and strengthened surveillance measures, our awareness of what is ethical is the decisive element. If authorship is a test, I hope every researcher can pass. Being an author of a paper, we share the glory, but we must also share the consequences.

Suqing Liu

Editorial Director
Kunming Institute of Zoology, Chinese Academy of Sciences
Kunming 650223*, China*

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Systematics of the Artiodactyla of China in the 21st century

Colin GROVES*

School of Archaeology & Anthropology, *Australian National University*, *Canberra*, *ACT* 2600, *Australia*

ABSTRACT

In this paper, I have introduced the concept of the Evolutionary Species, and shown how it affects the taxonomy of the Artiodactyla of China. The "traditional" taxonomy of the Artiodactyla, which has remained almost unchanged for 100 years, relies on ill-formulated notions of species and subspecies, only slightly modified by the population-thinking of the 1930s. Species are populations (or metapopulations) differentiated by the possession of fixed heritable differences from other such populations (or metapopulations). In the Artiodactyla, there are many more species than "traditionally" recognised; this is by no means a drawback, as it enables the units of biodiversity to be identified in a testable fashion, and brings the taxonomy of large mammals into line with that long practised for small mammals. Species are likely to differentiate where there are natural gaps in the distribution of a genus, such as mountain blocks (for example in the genus *Budorcas*) or otherwise dissected habitat (for example in the genus *Cervus*). Natural hybridisation between distinct species is not an uncommon phenomenon, again illustrated well in the genus *Cervus,* where hybridisation between the *elaphus* and *nippon* groups occurs today and evidently occurred in the past, as shown by the distribution of mtDNA.

Keywords: Artiodactyla; Taxonomy; China; Evolutionary species; Phylogenetic species concept

INTRODUCTION

Throughout the 20th century, the taxonomy of Artiodactyla which was in general use was based on Lydekker's *Catalogue of the Ungulate Mammals in the British Museum (Natural History)* (Lydekker, 1913-1916; Lydekker & Blaine, 1914). A few groups were revised during the 20th century, and a few changes were made, but always on the basis laid down in these volumes, so that Artiodactyla taxonomy became, as it were, "fossilised". It was only in 2011, with the publication of Groves & Grubb's *Ungulate Taxonomy*, that a new approach was laid down, bringing into play the advances in taxonomic and evolutionary understanding that had been accumulating since the 1980s (Groves & Grubb, 2011).

In the main, these advances in understanding concern species theory. Lydekker was influenced by the work of Rothschild and others who introduced the concept of the subspecies (or, as they were often called, "races") into mammalian taxonomy, but took it to extraordinary lengths. He wrote: "To a great extent the principal of classifying nearly related kinds of animals to races of a single species, rather than as distinct species, has been followed" (Lydekker, 1913, 1: vi). He did not say precisely what he meant by "nearly related kinds", nor did he define what he meant by species at all; evidently, he regarded species simply as convenient pigeonholes, and subspecies as lesser pigeonholes within them, neither being more than convenience categories.

The 1930s saw considerable advances in evolutionary theory, and it became clear that taxonomy should be based on populations. Mayr (1963) most explicitly defined species as populations (or groups of populations) which do not interbreed with other such populations (or groups of populations) under natural conditions, and the species/subspecies views of authors such as Lydekker fitted easily into this concept. Increasingly, however, systematists and evolutionists in general became dissatisfied with this definition, and proposed other concepts of species. Mayden (1997) reviewed all the concepts of species that had been proposed, and he concluded that only one fit in the criterion of a primary species concept: the "evolutionary species" (a species is an evolutionary lineage), arguing that what had been called the Phylogenetic Species Concept (that species are diagnosable - consistently different) is a suitable criterion for recognising evolutionary species. De Queiroz (2007) maintained that "every lineage is a species", and criteria such as non-interbreeding may be expected to evolve, over time, between different species, but again argued that the

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*Corresponding author, E-mail: colin.groves@anu.edu.au

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criterion of consistent difference is the most suitable way of differentiating them.

Groves & Grubb (2011: 1) therefore defined a species as "the smallest population or aggregation of populations which has fixed heritable differences from other such populations or aggregations". Such a definition means that species are units of taxonomy, and of biodiversity in general:

Species are populations (or groups of populations);

They have fixed differences between them (i.e., they differ 100%, consistently);

The differences between species are heritable (i.e., they relate at some level to the genome).

Species are thus testable, and any claim that two populations are diagnosable, i.e., different species, can be falsified if new evidence comes to hand. Morphological, genetic, and behavioural characters can all be used to diagnose species.

Many former subspecies, when closely examined, turn out to be fully diagnosable in this sense, and must therefore be regarded as distinct species. Those taxonomists who adhere to this view of species will thus recognise many more species than in the "traditional" view. Many of them do not recognise subspecies at all; others admit the subspecies as a taxonomic category, but agree that they are subjective, as they represent populations of a species which differ by the frequencies of certain characters, or by measurements which overlap. Whether subspecies are recognised or not, it is clear that they are not evolutionary units like species, and should therefore not be reified.

HIGHER CATEGORIES IN THE CLASSIFICATION OF ARTIODACTYLA

Since the 1990s, DNA-based phylogenetic research (see, for example, Waddell et al., 1999) has concluded that the old concept of Suiformes is inaccurate: of the three families formerly included in this group, the Suidae (pigs) and Tayassuidae (peccaries) are indeed evolutionary sister groups, but the Hippopotamidae (hippos) are more closely related to the ruminants, and their sister group is the Cetacea (whales and dolphins). This view of the hippopotamus as a sort of whale which remains partially terrestrial is revolutionary, but very solidly based.

The classification of the order Artiodactyla adopted by Groves & Grubb (2011) is as follows:

Suborder Tylopoda
- Family Camelidae

Suborder Suina
- Family Suidae
- Family Tayassuidae

Suborder Whippomorpha
- Infraorder Cetacea
- Infraorder Ancodonta
  - Family Hippopotamidae

Suborder Ruminantia
- Infraorder Tragulina
  - Family Tragulidae
- Infraorder Pecora
  - Family Moschidae
  - Family Giraffidae
  - Family Antilocapridae
  - Family Cervidae
  - Family Bovidae

The order contains most of our domesticated mammals. The nomenclature of domestic species and their wild ancestors/relatives has, in the past, been very contorted, but 10 years ago the International Commission on Zoological Nomenclature decreed that the name given to a domesticate shall not take precedence over that given to a wild species, and this was discussed by Gentry et al. (2004).

Tylopoda

The sole living family, Camelidae, consists of the genera *Camelus* and *Lama*. *Camelus ferus*, the wild Bactrian camel, is the only species native to China.

Suina

The genus *Sus*, the only genus of the pig family in China, is extremely difficult to classify. Provisionally, Groves & Grubb (2011) recognised three species in China: *Sus moupinensis* Milne Edwards, 1871, from most of China, extending south into Burma and north into Korea; *Sus chirodontus* Heude, 1888, from the swamps south-west of Shanghai; and *Sus ussuricus* Heude, 1888, from Heilongjiang and northern Jilin, extending into the Russian Far East.

Sus chirodontus is larger on average in males, and lighter-coloured, than *Sus moupinensis*, with a narrower skull, but females are the same size (i.e., there is less sexual dimorphism in size). *Sus ussuricus* is of enormous size in both sexes.

Moschidae

This is another family which is difficult to classify. Groves & Grubb (2011) recognised seven species, of which six are known from China:

Moschus moschiferus Linnaeus, 1758, the Siberian musk-deer, which extends from northernmost China (eastern Liaoning, eastern Jilin, north-eastern Nei Mongol, and Heilongjiang, extending to Korea, Mongolia and Siberia). This has a softer, less quill-like pelage than other musk-deer, with a pair of whitish stripes running from the chin to the chest, and the coat is often spotted.

Moschus chrysogaster Hodgson, 1839, the Alpine musk-deer, from the Alpine zone of the Tibetan Plateau, a very large species with an ill-defined broad whitish band running down the throat, pale rump, yellow-tipped ears, and light-coloured legs.

Moschus anhuiensis Wang et al., 1982, the Anhui musk-deer, from the lowlands of south-western Anhui, small and spotted.

Moschus berezovskii Flerov, 1929, the forest musk-deer, from the eastern slopes of the plateau, south into northernmost Vietnam, with three wide white stripes down the throat and black rump. There is some possibility that populations from southeastern China and northern Vietnam, which are much smaller and paler than others, might actually be a distinct species.

Moschus leucogaster Hodgson, 1839, the Himalayan musk-deer, from the southern slopes of the Himalayas, and

provisionally recorded in China in the Zhangmu district of south-eastern Tibet (unless this population, together with one in the Khumjung district of Nepal, represents a different species).

Moschus fuscus Li, 1981, the black musk-deer. There is a possibility that this species in fact consists of melanistic individuals of other species, but very dark musk-deer from the southern slopes of the eastern Himalayas, and brown musk-deer from northern Burma, are probably distinct species.

Recently, Liu & Groves (2014) published further notes on musk-deer, bringing together the rather disparate existing data and striking some cautionary notes. Pan et al. (2015) worked out a phylogeny of the genus, recognising the above six Chinese species and separating them into two clades: *M.chrysogaster*, *fuscus* and *leucogaster* versus *M. moschiferus*, *berezovskii* and *anhuiensis;* it is unclear where samples of *M. fuscus* and *leucogaster* derive from. A great deal remains to be learned about musk-deer taxonomy.

Cervidae

The two subfamilies of Cervidae are Capreolinae and Cervinae. The Capreolinae include the Capreolini (*Capreolus* and *Hydropotes*), Alceini (*Alces*) and Rangiferini (*Rangifer* and the New World dear, *Odocoileus* and its relatives). The Cervinae include the Muntiacini (*Elaphodus* and *Muntiacus)* and Cervini (*Dama, Axis, Rucervus, Panolia, Elaphurus* and *Cervus,* of which the last three occur in China*)*.

Two species of roedeer, *Capreolus*, are traditionally recognised (European *C. capreolus* and Asian *C. pygargus*), and Groves & Grubb (2011) had no evidence to affirm or query this, although there is some suggestion that *C. pygargus* may consist of two or three species. At present, it appears that the two species may overlap in the Caucasus, east of which *C. pygargus* extends as far as northern China, Korea and the Russian Far East; geographic populations of this presumed species differ in the number of B chromosomes, those from China (and other eastern areas) having more B chromosomes (up to 14) than those in Kazakhstan and western Russia.

The water-deer, *Hydropotes inermis*, is an antlerless deer which, according to molecular and behavioural datasets, is sister to roedeer. It is found in wetland areas of eastern China, mainly along the coast, especially Zhejiang, Jiangsu and the lower Yangtze, and in Korea. There is no evidence for more than one species, though this should be re-examined.

Alces americana is the moose (Elk) species found from central Siberia eastward through northern China and into North America, differing consistently from *Alces alces* of Europe and western Siberia. Populations from northern China and the Russian Far East appear distinctive, but it is unclear whether the differences are entirely genetic.

Reindeer, called caribou in North America, are traditionally ascribed to a single species, *Rangifer tarandus*, but some of the North American populations probably represent distinct evolutionary lineages, i.e., are distinct species. In the Old World, there are no distinctive Arctic island forms analogous to those in northern Canada, but the same tundra versus woodland division exists as in the New World. The relationships between the "ecotypes" in Eurasia (including far northern China) and North America have not been worked out, and a great deal of work remains to be done on the taxonomy of the genus.

Muntiacini

Traditionally, a single species, *Elaphodus cephalophus*, of Tufted Deer, has been recognised, from central and southern China (north as far as Sichuan, Hubei and Zhejiang) and northern Burma, but the mountainous distribution is probably divided into different blocks, and further research may find that several different evolutionary lineages (phylogenetic species) are involved. So far, insufficient material has been studied, and DNA work remains to be done.

There are many more species of the genus *Muntiacus*, known as muntjaks, than have previously been acknowledged. China has two species of the *M. muntjak* (Red or Indian muntjak) group. The first of these is the black-legged *M. nigripes* from Hainan, northernmost Vietnam and the southern mainland Chinese borderlands. The second is the pale-coloured *M. vaginalis*, which extends from Nepal and eastern India to Burma, Thailand, Laos and most of Vietnam into southernmost Yunnan, but the eastern populations are somewhat different from those of the South Asian regions, and might represent a different species. A third taxon, from Guangdong, has been described as *Muntiacus muntjak guangdongensis*, and needs to be compared with the two so far known from China. A second species-group is represented by *M. crinifrons* of western Zhejiang, south-eastern Anhui and Fujian, and *M. gongshanensis* of the Gaoligong and Biluo mountains. A third species-group is represented by *M. reevesi* which appears to have a wide distribution south of the Yangtze, including Taiwan.

Muntjak have long been known for their wide range of chromosome numbers. *M. reevesi* has a diploid chromosome number of 2*n*=46, the M. *crinifrons* group has eight in females and nine in males, and *M. vaginalis* has six in females and seven in males, the lowest number known in mammals.

Cervini

In most earlier publications, these typical Old World deer were generally referred to a single genus, *Cervus* (except for the distinctive *Dama, Axis* and *Elaphurus*), although sometimes the Sambar group have been set apart as a separate genus, *Rusa*, and the White lipped deer as genus *Przewalskium*. Work by Meijaard & Groves (2004) and by Pitra et al. (2004) has shown that relationships are different from those traditionally depicted: while *Elaphurus, Dama* (from western Eurasia) and *Axis* (from South and Southeast Asia) are indeed distinct, the only other distinct genera are *Panolia* and the mainly South Asian *Rucervus*.

One of the three species of *Panolia*, the Brow-antlered or Eld's deer, occurs in China. This is *P. siamensis*, a small spotted species which is rare in its mainland range (Thailand, Cambodia, Laos) but has been well-preserved on Hainan.

Père David's deer, *Elaphurus davidianus*, famously became extinct in the wild in historic times and preserved only in the Imperial Hunting grounds, whence a few specimens were

kidnapped in the 1890s and bred in large numbers in Britain. Many have now been returned to China, where they are breeding freely in much the same area as they were known in the 19th century. Characters of this peculiar genus, both morphological, behavioural and molecular, indicate it is of ancient hybrid origin between *Cervus* and *Panolia*.

There are numerous species in the genus *Cervus*, the "true deer", in China. *Cervus equinus*, the Southeast Asian sambar, has simple three-pointed antlers, a coarse, shaggy coat, and a long bushy tail. It occurs in Sumatra and Borneo, and mainland Southeast Asia north into southern China, including Taiwan.

Cervus albirostris, the White-lipped deer, is characteristic of the Tibetan Plateau.

Deer of the *C. elaphus* group – Red deer, wapiti, shou and sika – are widely distributed in China. In the far west, in the Tarim Basin in Xinjiang, occurs *C. yarkandensis*, characterised by a straight antler beam with only three tines. Both DNA and morphological data indicate that its affinities lie with deer related to *Cervus elaphus*, which are distributed in Europe and western Asia. In the Tianshan and Altai systems occurs the very different *C. canadensis*, identical (or nearly so) to the wapiti of North America, a very large deer with complex antlers and a very large rump patch; and a related species, *Cervus xanthopygus*, the Manchurian wapiti, occurs in north-eastern China and the Russian Far East. Three further species belong to the group known as the shou: *C. wallichii*, the Tibetan shou, in southern Tibet, along the Tsangpo River; *C. macneilli*, the Sichuan shou, on the wooded slopes of Sichuan and Gansu; and *C. alashanicus*, the Alashan stag, in Ninxia and the borders of Nei Mongol. These last three species have a characteristic antler form and small, dark-edged rump patches.

The last group of deer of the genus *Cervus* are related to *C. nippon*, the sika deer of Japan. They are much smaller than other species of the genus, and have very simple antlers. Different species of the group are known from the Japanese islands, while in China, *C. taiouanus* lives on Taiwan, *C. hortulorum* in northern China and the Russian Far East, *C. sichuanicus* in an isolated area of Sichuan, and *C. pseudaxis* in southeastern China and northern Vietnam. Unfortunately, throughout the Imperial period in China sika were kept on deer farms, and it seems likely that deer from different areas were mixed up, so that many surviving populations are of hybrid stock. There is still however scope to try to determine the affinities of deer from different captive facilities. There is a possibility that the full specific diversity of sika in China has not been documented; for example, it may be that the deer at present combined as *C. pseudaxis* from southern China and from northern Vietnam may actually represent two different species.

An interesting finding has been that the Chinese (and American) deer of the *C. elaphus* group, with the exception of *C. yarkandensis*, have mtDNA that aligns them with deer of the *C. nippon* group rather than with the European and West Asian representatives of the *C. elaphus* group. Groves & Grubb (2011) hypothesise that the *C. elaphus* group spread north-eastward from the southern Himalaya slopes, interbreeding with local sika populations as they spread, *elaphus*-group stags with *nippon*-group hinds, the pure stags then backcrossing with hybrid hinds over many generations until the deer population were entirely phenotypically *elaphus*-like but with *nippon*-like mtDNA.

Bovidae

The family Bovidae, the Hollow-horned Ruminants, is the largest family in the Order Artiodactyla, containing well over 200 species. This number is double that recognised before the Groves & Grubb (2011) revision; this has caused a certain amount of angst in some quarters (Heller et al., 2013; Zachos et al., 2013), but there have been cogent rebuttals (Cotterill et al., 2014; Groves, 2013). To put it very simply, we cannot complain about revisions to a taxonomic scheme that had remained unaltered, untested, for 100 years.

There are two subfamilies of Bovidae: Bovinae (containing cattle and buffaloes, as well as some Indian and African "antelopes"), and Antilopinae (containing sheep and goats and their relatives, gazelles, and a variety of African "antelopes"). The bovids occurring in China belong both to Bovinae (genus *Bos*) and to Antilopinae (genera *Saiga, Gazella, Procapra, Pantholops, Budorcas, Hemitragus, Pseudois, Capra, Ovis, Nemorhaedus, Capricornis*).

There appear to be no taxonomic problems connected with the Bovini. Two wild species of the genus *Bos* occur in China: *Bos gaurus*, the gaur, occurs in southernmost Yunnan, and *Bos mutus*, the wild yak, occurs on the Tibetan Plateau. Both have given rise to domestic forms: the mithan, derived from the gaur, is in use in northern Burma, north-eastern India and Bhutan, and possibly in southern Yunnan, while the domestic yak is spread throughout the Plateau and bordering mountains, at lower altitudes being hybridised with ordinary domestic cattle.

Antilopini

The saiga antelope, *Saiga tartarica,* was formerly widespread in Kazakhstan, and occurred in northern Xinjiang, but is believed to be now extinct in Chinese territory. The second, smaller species, *Saiga mongolica*, lives in western Mongolia and is not known to have occurred in China.

A single species of gazelle*, Gazella yarkandensis*, occurs in Xinjiang and probably other northern desert provinces of China. It has generally been regarded as a subspecies of *Gazella subgutturosa*, but this latter now appears to be confined to Iran and neighbouring countries.

All three species of the genus *Procapra* (formerly, but incorrectly, thought to be a kind of gazelle) live in Chinese territory. *Procapra picticaudata*, known as the Tibetan gazelle, the smallest species, is widespread across the Tibetan Plateau, just extending beyond Chinese territory into the Indian territory of Ladakh. The largest species, the Mongolian gazelle *Procapra gutturosa*, occurs in Nei Mongol.

It is the third species, Przewalski's gazelle *Procapra przewalskii*, which is of most interest. Its historical range was the region of Qinghai Lake, in the north-east of the Tibetan Plateau (*Procapra przewalskii przewalskii*), and lower altitudes in Gansu (*P.p diversicornis*). According to Mardan et al. (2013), it is now restricted to the shores of Qinghai Lake and the Buha River which flows into it, but the present population corresponds

to the putative subspecies *diversicornis,* and this has been so since about 1990 or before. Hypothetically, recent climatic change has caused the Gansu population to shift its range to higher altitudes, where it has outcompeted the indigenous population. To increase the complexity of the situation further, it appears that one of the surviving populations, in the Wayu region south-west of the lake, differs rather strongly morphologically from the others, a situation which needs further investigation (Mardan et al., 2013).

Caprini

The Chiru, *Pantholops hodgsoni*, is another species widespread on the Tibetan Plateau, and so almost confined to Chinese territory (just extending into Ladakh).

The takins (genus *Budorcas*) have traditionally been ascribed to a single species, but it is unclear why, since on the available evidence the four named taxa appear to be consistently different from each other, and so represent unique evolutionary species (Groves & Grubb, 2011). All four occur in China, two of them being endemic. *Budorcas taxicolor,* extending from the Mishmi Hills of north-eastern India through northernmost Burma into Yunnan, is light brown developing with age an extensive black wash on the flanks, belly, legs, throat and head; males are much larger than females. *Budorcas whitei* ccurs in south-eastern Tibet and in Bhutan; in skull, horns and pelage it resembles juveniles of *B. taxicolor*, but is much the same size (an apparent case of neoteny). *Budorcas thibetana*, from Sichuan, lacks the specific dark areas of *B. taxicolor* but develops overall darkening with age and a yellowish saddle on the back, and with a black nose. Finally the beautiful Golden takin, *Budorcas bedfordi*, from the Qinling Range, is more golden in tone, without darkening on the face, and is less sexually dimorphic than other species.

A genus that is distributed over discontinuous habitat, such as high altitude grasslands and forests, like *Budorcas*, is especially likely to undergo speciation. Most genera of the tribe Caprini, almost all of which are specialists of high altitude or hilly country, are divided into well marked divergent species in this manner.

The only species of true wild goat (*Capra*) in China is the so-called Siberian ibex, *Capra sibirica*, which is spread from Kashmir to the Altai and the Gobi ranges, and enters Chinese territory in Xinjiang, Gansu and Nei Mongol. Geographic variation in this species, or whether it is indeed a single species, remains to be worked out properly.

The genus *Ovis*, containing sheep (domestic and wild), is widespread from the Middle East across central and northern Asia into North America. The number of species has been much debated; most authors have divided the Old World wild sheep into urial (from the lower desert ranges of the Middle East), argali (from the high mountains of Central Asia), and snow sheep (from central and eastern Siberia), treating these as species, or sometimes subspecies-groups within a single species. In the analysis of Groves & Grubb (2011), each of these informal groups contains a number of species. Only argali are found in China. *Ovis hodgsoni*, the Tibetan argali, spread over the whole of the Tibetan Plateau, is a large, mainly uniformly brown species with a sharply set-off light belly and rump and a long light-coloured “ruff” on the throat. In the Tianshan occurs the so-called Marco Polo sheep, *Ovis polii*, which is smaller but with very long, widely spreading horns; it is darker, with a much more spreading pale rump patch and a very small ruff present only in winter pelage. *Ovis darwini*, the Gobi argali, may extend into Chinese territory; the Shanxi argali, *Ovis jubata*, is endemic to China (Shanxi, Shaanxi, Hebei, Nei Mongol); it is small and dark fawn-grey in colour with a large rump patch, and a very long ruff.

The Gorals, genus *Nemorhaedus*, live on wooded slopes up to about 3 000 m., from the southern slopes of the Himalayas south-east to northern Thailand and north-east to the Russian Far East. The Chinese goral, *Nemorhaedus griseus*, is endemic to China, from Fujian west to Sichuan and north to Shanxi and (formerly) the vicinity of Beijing; it is grey in colour, light on underparts, with yellowish or white limbs below knees and hocks, and a golden-white throat; the tail is bushy but short. To the north-east (Heilongjiang, extending west to Mongolia and north to Korea and the Russian Far East) occurs the Long-tailed goral*, Nemorhaedus caudatus*, pale in colour, with no golden tone on the throat, and an extremely long tail-brush. To the south (Yunnan, extending to Burma and Thailand) occurs the Burmese goral, *Nemorhaedus evansi*, very light fawn in colour, and at least in summer very short-coated. Finally at higher altitudes in south-eastern Tibet, the Gongshan, and far northern Burma occurs the Red goral, *Nemorhaedus baileyi*, distinguished by its foxy-red colour with pale underparts and small size.

The final genus of Chinese Caprini is *Capricornis*, the serows, whose total range extends from Sumatra to Gansu and Shaanxi and west to the southern slopes of the Himalayas, in hilly country but mostly not excessively high altitudes. Endemic to China is the White maned serow, *Capricornis milneedwardsi*, which is black, with whitish and reddish lights, legs red below the knee and hock, and a long silvery-white mane. Just entering Chinese territory in the Chumvi Valley (Tibet, on the borders of India and Bhutan) is the Himalayan serow, *Capricornis thar*, with a mane that has less white in it, and the legs are white rather than reddish below the knees. The brindled-toned Indochinese serow*, Capricornis maritimus,* may just enter Chinese territory to the north of the Vietnam border.

OVERVIEW

The species of Artiodactyla occurring in China are listed in Table 1.

The realisation that a species is an evolutionary lineage makes explicit an undercurrent that has pervaded the taxonomy of small mammals for several decades. When applied to small mammals, such as rodents, insectivores and bats, it is relatively uncontroversial, but the idea that this is the essence of species also in large mammals has met with some resistance (see above in the introductory section). Looked at in this way, it can be seen that species (unlike genera, families and so on) have a real existence. They are defined not as having “enough difference” from their relatives to qualify as species, but as being objective units of biodiversity. If it means that there are twice as many species of Bovidae and other artiodactyls as we have thought, 10 years ago, this is a small price to pay for objectivity.

Table 1 List of species of living Artiodactyla occurring in China

Species	Common name	Notes
Camelus ferus	Wild Bactrian camel	
Sus moupinensis	Common Chinese wild pig	Needs further revision
Sus chirodontus	Chinese marshlands wild pig	
Sus ussuricus	Ussuri wild pig	
Moschus moschiferus	Siberian musk-deer	
Moschus chrysogaster	Alpine musk-deer	
Moschus leucogaster	Himalayan musk-deer	Possibly an undescribed species
Moschus anhuiensis	Anhui musk-deer	
Moschus fuscus	Black musk-deer	Status doubtful
Moschus berezovskii	Forest musk-deer	Possibly more than one species
Capreolus pygargus	Asian roedeer	Possibly more than one species
Hydropotes inermis	Chinese water deer	Taxonomic revision needed
Alces americana	Eastern moose	
Rangifer tarandus	Reindeer	Taxonomic revision needed
Elaphodus cephalophus	Tufted deer	Possibly more than one species
Muntiacus nigripes	Black-footed muntjak	
Muntiacus vaginalis	Indian Red muntjak	Taxonomic revision needed
Muntiacus crinifrons	Hairy-fronted muntjak	
Muntiacus gongshanensis	Gongshan muntjak	
Muntiacus reevesi	Chinese muntjak	
Panolia siamensis	Eastern Eld's deer	
Elaphurus davidianus	Père David's deer	
Cervus equinus	Sambar	Taxonomic revision needed
Cervus albirostris	White-lipped deer	
Cervus yarkandensis	Yarkand deer	
Cervus canadensis	Wapiti	
Cervus xanthopygus	Manchurian wapiti	
Cervus wallichii	Wallich's deer	
Cervus macneilli	McNeil's deer	Further research needed
Cervus alashanicus	Alashan deer	Further research needed
Cervus hortulorum	North Chinese sika	
Cervus taioanus	Formosan sika	
Cervus sichuanicus	Sichuan sika	
Cervus pseudaxis	Vietnam sika	Taxonomic revision needed
Bos gaurus	Gaur	
Bos mutus	Wild yak	
Saiga tatarica	Saiga	Probably extinct in China
Gazella yarkandensis	Yarkand gazelle	
Procapra picticaudata	Tibetan gazelle	
Procapra przewalskii	Przewalski's gazelle	Further research needed
Procapra gutturosa	Mongolian gazelle	
Pantholops hodgsoni	Chiru	

Continued

Species	Common name	Notes
Budorcas taxicolor	Mishmi takin	
Budorcas whitei	Bhutan takin	
Budorcas thibetana	Sichuan takin	
Budorcas bedfordi	Golden takin	
Capra sibirica	Siberian ibex	
Ovis hodgsoni	Tibetan argali	
Ovis polii	Marco Polo sheep	
Ovis jubata	Shanxi argali	
Ovis darwini	Gobi argali	Not certainly existing in China
Nemorhaedus griseus	Chinese grey goral	
Nemorhaedus caudatus	Longtailed goral	
Nemorhaedus evansi	Burmese goral	
Nemorhaedus baileyi	Red goral	
Capricornis milneedwardsi	White maned serow	
Capricornis thar	Himalayan serow	May occur in China
Capricornis maritimus	Indochinese serow	May occur in China

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Comparative transcriptome analysis on the alteration of gene expression in ayu (*Plecoglossus altivelis*) larvae associated with salinity change

Xin-Jiang LU[1], Hao ZHANG[1], Guan-Jun YANG[1], Ming-Yun LI[1], Jiong CHEN[1,2,*]

[1] *Laboratory of Biochemistry and Molecular Biology*, *School of Marine Sciences*, *Ningbo University*, *Ningbo* 315211, *China*

[2] *Collaborative Innovation Center for Zhejiang Marine High-Efficiency and Healthy Aquaculture*, *Ningbo University*, *Ningbo* 315211, *China*

ABSTRACT

Ayu (*Plecoglossus altivelis*) fish, which are an amphidromous species distributed in East Asia, live in brackish water (BW) during their larval stage and in fresh water (FW) during their adult stage. In this study, we found that FW-acclimated ayu larvae exhibited a slower growth ratio compared with that of BW-acclimated larvae. However, the mechanism underlying FW acclimation on growth suppression is poorly known. We employed transcriptome analysis to investigate the differential gene expression of FW acclimation by RNA sequencing. We identified 158 upregulated and 139 downregulated transcripts in FW-acclimated ayu larvae compared with that in BW-acclimated larvae. As determined by Gene Ontology annotation and Kyoto Encyclopedia of Genes and Genomes pathway mapping, functional annotation of the genes covered diverse biological functions and processes, and included neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. Transcriptional expression of several differentially expressed genes in response to FW acclimation was further confirmed by real-time quantitative PCR. In accordance with transcriptome analysis, *iodothyronine deiodinase* (*ID*), *pro-opiomelanocortin* (*POMC*), *betaine-homocysteine S-methyltransferase 1* (*BHMT*), *fructose-bisphosphate aldolase B* (*aldolase B*), *tyrosine aminotransferase* (*TAT*), and *Na^+-K^+ ATPase* (*NKA*) were upregulated after FW acclimation. Furthermore, the mRNA expressions of *b-type natriuretic peptide* (*BNP*) and *transgelin* were downregulated after FW acclimation. Our data indicate that FW acclimation reduced the growth rate of ayu larvae, which might result from the expression alteration of genes related to endocrine hormones, energy metabolism, and direct osmoregulation.

Keywords: *Plecoglossus altivelis*; Salinity change; Transcriptome analysis; Growth rate; Real-time quantitative PCR

INTRODUCTION

A variety of fish undergo migration to cope with environmental fluctuations, such as temperature variation and food availability (Jorgensen & Johnsen, 2014). Some fish species migrate seaward after hatching to grow in marine habitats, and then migrate riverward to further develop in freshwater. This type of diadromy is categorized as amphidromy (McDowall, 1992). Ayu (*Plecoglossus altivelis*), the only member in the genus *Plecoglossus* of the family *Plecoglossidae*, is an amphidromous fish distributed in East Asia. After hatching in estuaries, ayu larvae migrate to the sea where they spend the winter. In spring, they move to the middle reaches of rivers, before returning to estuaries in autumn where they spawn and die. Hence, osmoregulation in ayu seems to be related to their growth and development; however, it is still unclear which genes mediate osmoregulation and growth of ayu larvae.

The growth rates of teleosts can be affected by salinity (Gillanders et al., 2015; Schwarz & Allen, 2014). Salinity induced-change in growth rates mainly results from standard metabolic rates and hormonal stimulation (Boeuf & Payan, 2001). Fish can acclimate to different salinity conditions during migration by several osmoregulatory mechanisms. Osmoregulation occurs in two consecutive phases: (1) an initial period with changing osmotic variables; and (2) a chronic regulatory period during which the variables reach new homeostasis (Holmes & Donaldson, 1969). During these two phases, altered gene expressions play an important role (Weng et al., 2002).

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[*]Corresponding author, E-mail: jchen1975@163.com

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Changes in the expression of *Na^+-K^+ ATPase* (NKA) in teleosts, for example, provide the primary driving force for the operation of different ion transporters according to environmental salinity (Hwang & Lee, 2007; Kang et al., 2015). In addition to affecting the ion pump, fish respond to changes in salinity with compensatory acclimations that also include regulating hormones (Peter, 2011; Sakamoto & McCormick, 2006; Yada et al., 2010), re-establishing osmotic homeostasis (Hwang & Lee, 2007), altering cell structure and function (Avella et al., 2009), and changing energy metabolism (Tseng & Hwang, 2008). The regulating genes of the above physiological processes are directly or indirectly implicated in the change in growth rates. However, gene expression alteration is dependent on species after acclimation to different osmotic stresses (Tseng & Hwang, 2008).

Transcriptome sequencing technologies are a powerful and cost-effective approach in the application of teleost investigation. Several economically or scientifically important fish species have been analyzed at the transcriptome level, including grass carp (*Ctenopharyngodon idellus*), Atlantic salmon (*Salmo salar* L.), and rainbow trout (*Oncorhynchus mykiss*) (Salem et al., 2010; Tian et al., 2015; Wang et al., 2014). Development of transcriptome sequencing in these species provides access to functional and evolutionary analyses previously restricted to genetic model organisms.

Ayu is a popular and highly valued edible fish in East Asia. In industrial aquaculture, ayu larvae are incubated in brackish water (BW) for eight weeks after hatching and then transferred to fresh water (FW) for further growth. In this study, we found that BW-acclimated ayu larvae grew faster than FW-acclimated larvae. Furthermore, we sequenced the transcriptome of a variety of tissues in ayu larvae acclimated to BW or FW. We found that FW acclimation altered the expression of genes mainly implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton.

MATERIALS AND METHODS

Fish and experimental conditions

Ayu larvae were kept in BW or FW tanks at 20-22 °C in a recirculating system with filtered water after hatching, and were fed with commercial pellets once a day. Groups containing 20 fish each were subjected to BW (containing 10 mg/mL) in four tanks (BW groups) or to FW in four tanks (FW groups).

Tissue sampling and sequencing

Heart, liver, spleen, gill, kidney, skin, muscle, intestine, and brain tissues from ayu after BW or FW acclimation for four weeks were mixed. Total RNA was extracted using TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The extracted RNA was treated with DNase I for 30 min at 37 °C (New England BioLabs, Beverly, MA, USA) to remove residual DNA. Oligo (dT) beads were used to isolate mRNA. After the mRNA was fragmented as templates, the cDNA first-strands were synthesized using random hexamer-primer and reverse transcriptase (Invitrogen). Second-strands were synthesized using RNaseH (Invitrogen) and DNA polymerase I (New England BioLabs). Paired-end libraries with average insert lengths of 200 base pairs (bp) were synthesized according to Illumina (CA, USA) protocols. Transcriptome sequencing of ayu macrophages was performed at the Beijing Genomics Institute (BGI) in Shenzhen, China, using the Illumina HiSeq^{TM} 2000 platform. Transcriptome data were deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE73321.

Assembly and analysis of differential expression

Data quality control and sequence filtering of repetitive, low-complexity, and low-quality reads prior to assembly of sequenced reads for non-redundant consensus were performed using the FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and PRINSEQ software (Schmieder & Edwards, 2011). The default parameters were applied when using Trinity (Grabherr et al., 2011) for first assembly, and parameters "-new_ace -minmatch 12 -minscore 20 -repeat_stringency 0.9" were set for the Phrap software (Ewing & Green, 1998). The Markov Cluster Algorithm (MCL, http://micans.org/mcl, Stijn van Dongen) was performed as follows. First, similarity was detected using Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with more than 90% identity and more than 60% coverage were filtered out. Subsequently, the similar sequences were used by the TRIBE_MCL algorithm. The longest sequences were selected as the representative transcripts. Gene expression levels were estimated by mapping clean reads to a reference set of assembled transcripts using RSEM (Li & Dewey, 2011). FPKM (fragments per kilobase of exon model per million mapped reads) was used as the value of gene expression levels (Trapnell et al., 2010). Differential expression was assessed using DESeq (Anders & Huber, 2010). Genes with fold change >2 or <0.05 and P<0.001 indicated significant expression abundance.

BLAST against sequence databases and functional annotation

The homology of transcriptome sequences was searched using the BLASTX program against sequences in the NCBI non-redundant (NR) protein database and in the SwissProt database (E-value<1e-5). Genes were tentatively identified according to the best hits against known sequences. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed using DAVID software (Huang et al., 2009).

Real-time quantitative PCR (RT-qPCR)

The ayu samples were collected and preserved at -80 °C after BW or FW acclimation. Total RNA was extracted and purified from ayu mixed tissue samples. After deoxyribonuclease I treatment, the isolated RNA was reverse transcribed with M-MLV Reverse Transcriptase (TaKaRa, Dalian, China). Specific primer pairs of selected genes were designed (Table 1). The reaction mixture was incubated for 300 s at 95 °C, followed by 39 amplification cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, in a StepOne-Real Time PCR platform (Applied Biosystems, Foster City, CA, USA). The threshold cycle data

(Ct) and baselines were determined using auto settings. Ct values of genes for ayu macrophage samples were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method, as previously described (Huang et al., 2011; Livak & Schmittgen, 2001).

Table 1 Oligonucleotide primers used to amplify cDNA of selected ayu genes

Gene	Primer	Nucleotide sequence (5′-3′)	Accession number or transcript name
BHMT	BHMT(+)	GCAACAGCCTTAGCTTCACA	FN561754
	BHMT(-)	CTGTCTCACTCTTACAGCTC	
BNP	BNP(+)	CGACGGACTGATGACAAATG	comp127789_c0_seq1
	BNP(-)	TCTGCGGTTGTCCTTTCTCT	
Aldolase B	Aldolase B(+)	CACGAGACGCTGTACCAGAA	comp51413_c0_seq1
	Aldolase B(-)	TTCAGAACGCTCCTCCACTT	
ID	ID(+)	CGCCTACAAGCAGGTAAAGC	comp51414_c0_seq1
	ID(-)	GGCGGTCTGAAGACTCAAAG	
POMC	POMC(+)	ACAGCCAGCAGGAGAAGAAG	comp116637_c0_seq1
	POMC(-)	CTGCTGTCCGTTCTTGTTGA	
NKA	NKA(+)	AGAGTGATGTGGGGATCAGG	comp113388_c2_seq1
	NKA(-)	AATGCTCCCGTTTGTGGTAG	
Transgelin	Transgelin(+)	GCCTGGCAGTCACTAAGGAG	comp106110_c0_seq1
	Transgelin(-)	TCCGTAGCTCATACCCTGCT	
TAT	TAT(+)	GCTTCCTCAAGTCCAACTCG	comp117135_c0_seq1
	TAT(-)	CGATGCTGGAAGACAGAACA	
β-actin	β-actin (+)	TACCGGTTGGTACATCAGCA	AB020884
	β-actin (-)	TGACGGTAAAGTTGGTGCAA	

Statistical analysis

All data are shown as means±*SEM*. Data were further analyzed by one-way analysis of variance (ANOVA) using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). In all cases, $P<0.05$ was considered statistically significant.

RESULTS

Growth after BW or FW acclimation

The length and body weight of ayu larvae were recorded to assess growth status after BW or FW acclimation. Three weeks after hatching, there was no significant change in ayu larvae length (Figure 1A). After four weeks, however, ayu larvae acclimated to BW were significantly longer than those acclimated to FW (Figure 1A). After eight weeks, the length of larvae acclimated to FW was only 72.3% that of the larvae acclimated to BW (Figure 1A). Two weeks after hatching, there was no significant change in ayu larvae body weight (Figure 1B). After three weeks, however, ayu larvae acclimated to BW were significantly heavier than those acclimated to FW (Figure 1B). After eight weeks, the body weight of larvae acclimated to FW was only 41.1% that of the larvae acclimated to BW (Figure 1B).

Transcriptome sequencing and assembly

Two cDNA libraries obtained from the poly(A)-selected RNAs of BW- or FW-acclimated ayu tissue samples were sequenced using the Illumina HiSeq™ 2000 platform, and approximately 79.7 (BW) and 79.2 (FW) million 90-bp pair end (PE) reads were generated. Phrap software was applied successively to improve assembly accuracy in the presence of repeats. Through assembly and elimination of redundancy, 172 623 contigs were generated from both libraries with an average length of 1 319 bp, and an N50 of 2 292 bp. The distribution of contig length is shown in Figure 2, and varied from 201 bp to more than 2 000 bp. According to the P-values, comparison of read occurrences between the two libraries revealed that 297 unique tags were differentially represented between the libraries (absolute value of fold change>2, composed of 139 contigs more represented in the BW library and 158 contigs more represented in the FW library).

Functional annotation based on GO and KEGG analysis

Starting with a similarity search against the main protein and nucleotide sequence databases, annotated matches were obtained from the Swiss-Prot database (36 203 unigenes) and the NR database (43 128 unigenes). To assess their evolutionary conservation, the 43 128 unigenes mapped to the NR database were searched against the sequences of other fish species in the database using the BLASTx algorithm (Figure 3). Overall, 23.5% of the NR-annotated unigenes were identified in *Maylandia zebra* and 18.6% were identified in *Oreochromis niloticus*.

Subsequent to the above analyses, the differentially expressed sequences were functionally classified using the Blast2Go program. GO terms were assigned to 297 unigenes that belonged to biological processes, cellular components, and molecular function clusters, and were distributed among more

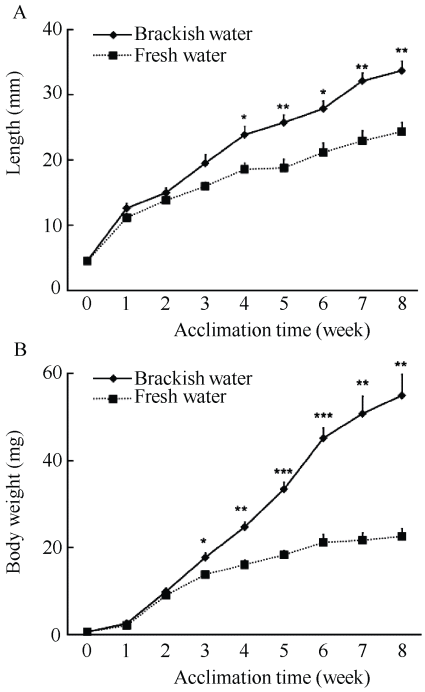

Figure 1 Effect of BW and FW acclimation on length (A) and body weight (B) of ayu larvae

Ayu larvae were acclimated to BW or FW for 8 weeks after hatching. Lengths and body weights were recorded weekly. Bars represent means± *SEM* of four biological replicates. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

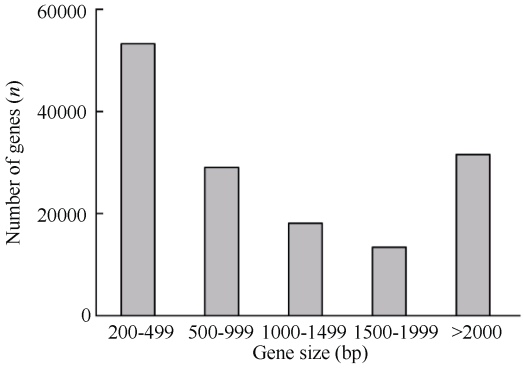

Figure 2 Summary distribution of the lengths of the assembled contigs

The final contig set contained 145 445 contigs with an N50 of 2 292 bp (⩾200 bp, mean length=1 319 bp, max length=19 352 bp)

than 35 categories (Figure 4), including metabolism, development, apoptosis, response to stimulus, and the immune system. Among them, 62 and 14 unigenes were assigned to GO terms "metabolic process" and "response to stimulus", respectively. The *P*-values of GO terms are listed in Table 2.

To investigate the overall biological function of differentially expressed genes, we assigned the unigenes based on the KEGG pathway. A total of 297 unigenes were assigned to 21 known metabolic or signaling pathways (Figure 5). Eight pathways were associated with energy metabolism, as well as the nervous and endocrine systems, including cofactor and

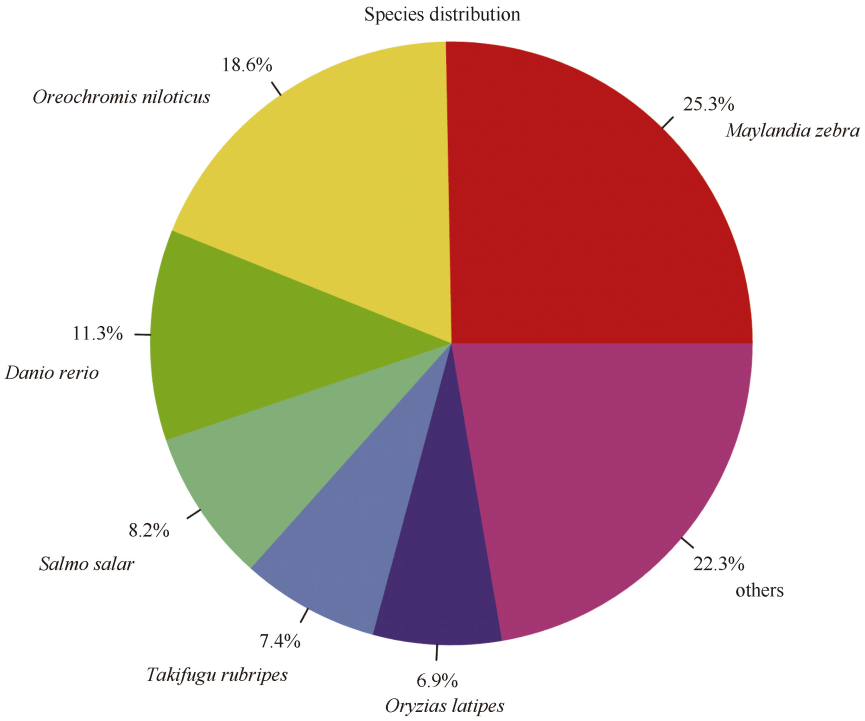

Figure 3 Percentages of the ayu unigenes conserved in other fish species

Unigenes were searched against genomic sequences of other fish species in the NR database using the BLASTx algorithm.

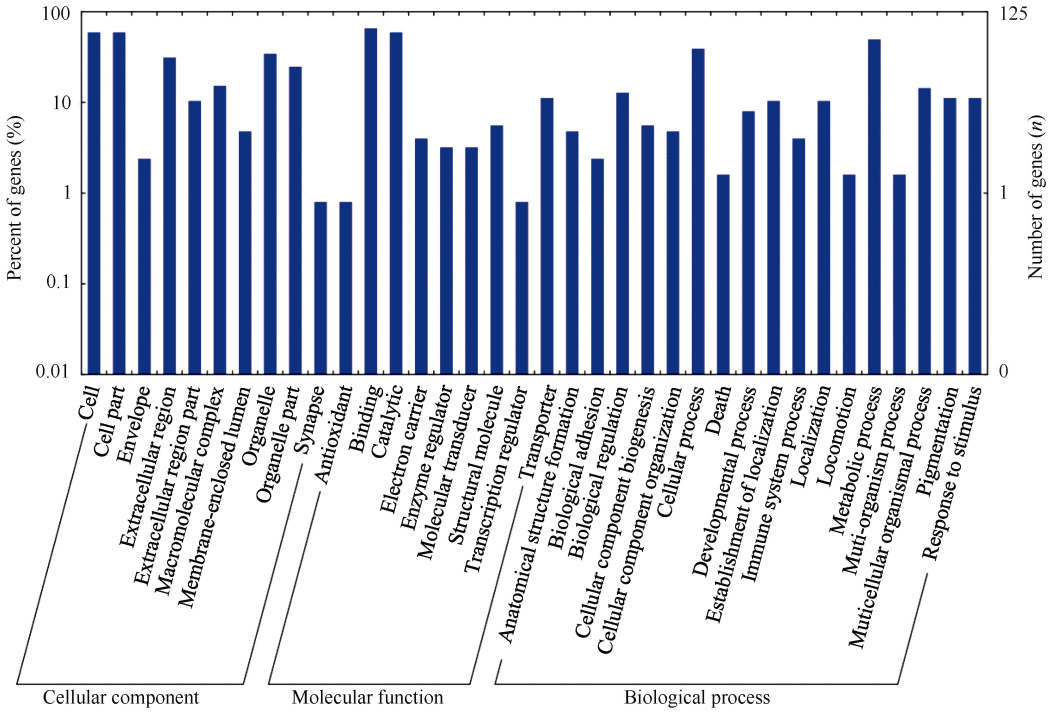

Figure 4 GO classification of differentially expressed genes

Transcripts were annotated by GO terms, and belonged to three main categories: biological processes, cellular components, and molecular functions.

Table 2 *P*-values of GO terms

Ontologies	GO term	*P*-value
Cellular Component	Cell	1
	Cell part	7.79E-05
	Envelope	0.876383
	Extracellular region	1.00E-15
	Extracellular region part	0.000665
	Macromolecular complex	0.687542
	Membrane-enclosed lumen	0.983933
	Organelle	1.33E-05
	Organelle part	0.957311
	Synapse	0.992793
Molecular Function	Antioxidant	0.20312618
	Binding	0.97707844
	Catalytic	8.44E-145
	Electron carrier	0.008248341
	Enzyme regulator	0.96959837
	Molecular transducer	2.19E-08
	Structural molecule	0.124043728
	Transcription regulator	0.999432387
	Transporter	0.171271508
Biological Process	Anatomical structure formation	0.937536

Continued

Ontologies	GO term	*P*-value
Biological Process	Biological adhesion	0.977677
	Biological regulation	1
	Cellular component biogenesis	0.750927
	Cellular component organization	0.999999
	Cellular process	1
	Death	0.996138
	Developmental process	0.999982
	Establishment of localization	0.000894
	Immune system process	0.52067
	Localization	0.99828
	Locomotion	0.928613
	Metabolic process	0.032464
	Multi-organism process	0.000108
	Multicellular organismal process	0.999769
	Pigmentation	1
	Response to stimulus	0.00449

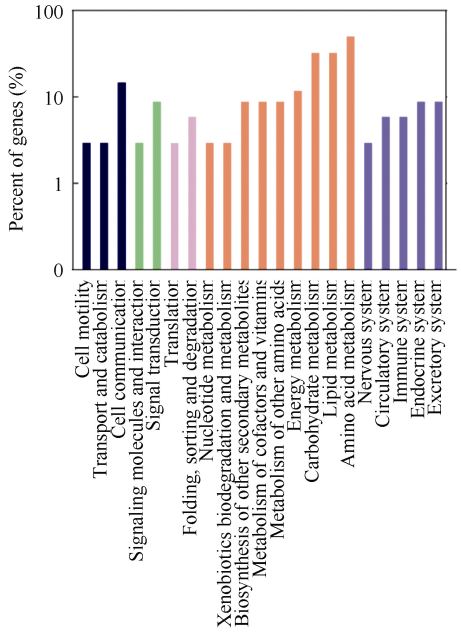

Figure 5 Functional distribution of differentially expressed genes based on KEGG analysis

vitamin metabolism, other amino acid metabolism, energy metabolism, carbohydrate metabolism, lipid metabolism, amino acid metabolism, and nervous and endocrine system processes.

Profiling gene expression patterns

We further analyzed differentially expressed genes related to salinity acclimation. Table 3 shows genes selected due to their responsiveness in the transcriptome, and which were determined to be involved in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. The transcriptional expression of several differentially expressed genes in response to salinity acclimation was further confirmed by RT-qPCR (Figure 6) at different time points. In accordance with the transcriptome analysis, data indicated that mRNA expression of *BHMT*, *aldolase B*, *ID*, *POMC*, *NKA*, and *TAT* were upregulated after FW acclimation (Figure 6); *BHMT*, *aldolase B*, and *TAT* were upregulated 3 and 4 weeks after FW acclimation; *ID* and *POMC* were upregulated at all time points; and, *NKA* was upregulated 2, 3, and 4 weeks after FW acclimation. The mRNA expressions of *BNP* and *transgelin* were downregulated after FW acclimation (Figure 6); *BNP* was downregulated at all time points; and, *transgelin* was downregulated 3 and 4 weeks after FW acclimation.

DISCUSSION

Salinity, an important environmental factor, influences the growth of fish (Boivin et al., 2015). We therefore investigated the mechanism underlying the effect of salinity on the growth of ayu. Ayu larvae live in BW initially, but move to FW habitats after several months. In this study, we found that BW-acclimated ayu larvae had a higher growth rate compared with that of FW-acclimated larvae. Furthermore, transcriptome sequencing was employed to measure changes in ayu larvae gene expression associated with changes in environmental salinity. Genes with significant expression alterations were implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. This suggests that growth suppression in FW-acclimated larvae resulted from alteration in the expression of several genes associated with endocrine hormones, osmotic regulation, and energy metabolism.

Table 3 Selected differentially expressed genes as determined by transcriptome sequencing

Transcript name	Fold change (FW/BW)	Identity	*P*-value
Neuroendocrinology			
comp116637_c0_seq1	Only FW	*Pro-opiomelanocortin*	1.03E-08
comp51414_c0_seq1	12.846	*Iodothyronine deiodinase*	3.58E-16
comp127789_c0_seq1	0.242	*B-type natriuretic peptide*	2.78E-17
comp87338_c0_seq1	5.366	*Neurogranin*	1.78E-04
Osmotic regulation			
comp127693_c0_seq1	3.122	*Betaine--homocysteine S-methyltransferase 1*	0
comp108643_c0_seq1	2.174	*Inositol oxygenase*	1.68E-04
comp87233_c0_seq1	2.115	*Allantoinase*	5.35E-07
comp113388_c2_seq1	2.096	*Sodium potassium ATPase beta*	5.51E-08
comp117155_c0_seq1	0.277	*Ammonium transporter*	5.32E-11
Energy metabolism			
comp107204_c0_seq1	2.105	*4-hydroxyphenylpyruvate dioxygenase*	1.57E-04
comp117135_c0_seq1	2.315	*Tyrosine aminotransferase*	5.79E-08
comp51385_c0_seq1	3.125	*Homogentisate 1, 2-dioxygenase*	5.34E-19
comp117911_c0_seq1	0.048	*Phospholipase A2*	1.63E-05
comp127763_c0_seq1	2.482	*Fatty acid-binding protein*	3.60E-09
comp51523_c0_seq1	3.136	*Alkylglycerol monooxygenase*	1.22E-06
comp51413_c0_seq1	2.635	*Fructose-bisphosphate aldolase B*	0
comp119933_c0_seq2	2.197	*mitochondrial uncoupling protein 2*	6.87E-05
comp123015_c0_seq1	Only FW	*Vacuolar ATP synthase subunit F*	4.19E-06
Cytoskeleton			
comp118771_c0_seq1	0.304	*Scinderin*	9.17E-08
comp106110_c0_seq1	0.292	*Transgelin*	6.07E-07
comp112167_c1_seq1	0.325	*Myosin heavy chain*	5.84E-12
comp120851_c4_seq2	0.454	*Myosin-1*	6.17E-08
comp123726_c0_seq2	0.358	*Titin*	1.15E-04

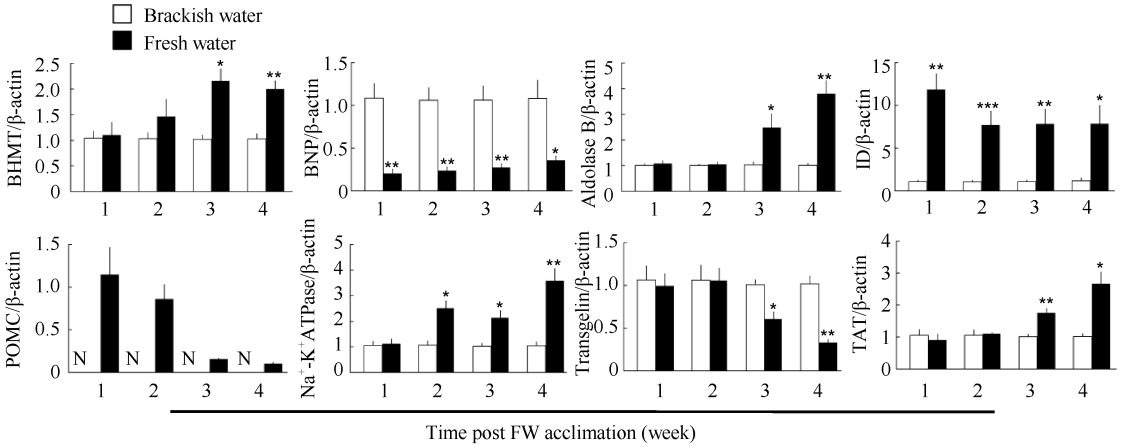

Figure 6 RT-qPCR confirmation of differential gene expression in BW- or FW-acclimated ayu

RNA was isolated from ayu multi-tissue samples and RT-qPCR was performed 1, 2, 3, and 4 weeks after BW or FW acclimation. β-actin was used for normalization. Bars represent means±*SEM* of four biological replicates. N: not detected. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

Ayu, an aquacultured fish species, has been previously employed to investigate the immune system in teleosts (Chen et al., 2014; Lu et al., 2013). However, the mechanism underlying salinity acclimation is still poorly known. Hence, we used tissue-mixed samples from FW- and BW-acclimated ayu to detect differentially expressed genes by transcriptome sequencing. Tissue-mixed samples have also been used in other aquaculture fish to investigate gene expression by transcriptome sequencing (Xiao et al., 2015). Although transcriptome comparison of tissue-mixed samples can result in lost information underlying acclimation and certain measurement errors, we found 297 differentially expressed genes between FW- and BW-acclimated ayu. These genes were mainly implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton, which are all related to salinity acclimation. Hence, transcriptome analysis of tissue-mixed samples in this study successfully found several possible mechanisms underlying ayu salinity acclimation.

Endocrine hormones regulate a variety of physiological processes, including stress, growth, energy metabolism, and osmoregulation. In this study, FW acclimation led to the upregulation of three genes in the endocrine hormone system, that is, *POMC*, *ID*, and *BNP*. Moreover, the expression of these genes changed 1 week after FW acclimation, earlier than genes implicated in energy metabolism and osmotic regulation. These results suggest that these gene-related hormones may control osmotic regulation and energy metabolism. POMC can be cleaved to give rise to three peptide hormones, α-melanocyte stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), and β-endorphin (Böhm & Grässel, 2012). α-MSH has important roles in the regulation of appetite and sexual behavior. ACTH enhances the secretion of glucocorticoids from the adrenal cortex to regulate stress behavior. β-endorphins are endogenous opioid peptides with widespread action in the brain, including reward and pain. Mammalian α-MSH is a tridecapeptide cleaved from POMC that acts to inhibit food intake (Ramos et al., 2005). In the goldfish hypothalamus, α-MSH has also been implicated in constraining food consumption (Shimakura et al., 2008). The upregulated POMC in ayu larvae after FW acclimation might induce food intake suppression and thus inhibit growth. ACTH can stimulate gill NKA activity in juvenile teleosts (Langdon et al., 1984). Transcriptome sequencing and RT-qPCR data showed that the mRNA expression of *NKA* was upregulated in FW-acclimated ayu larvae. Since NKA will cost ATP, FW acclimation could increase energy consumption to induce growth suppression in ayu larvae.

FW acclimation in ayu larvae also upregulated a variety of genes related to energy metabolism, such as *4-hydroxyphenylpyruvate dioxygenase*, *TAT*, *fatty acid-binding protein*, *alkylglycerol monooxygenase,* and *aldolase B*. TAT can catalyze the conversion of tyrosine to 4-hydroxyphenylpyruvate, which is converted to homogentisate by 4-hydroxyphenylpyruvate dioxygenase. Hence, the mRNA expressed upregulation of *4-hydroxyphenylpyruvate dioxygenase* and *TAT* may promote tyrosine catabolism. Both fatty acid-binding protein and *alkylglycerol monooxygenase* were upregulated in FW-acclimated ayu larvae. The fatty-acid-binding protein is in charge of fatty acid transport, while alkylglycerol monooxygenase can catalyze the hydroxylation of alkylglycerol, which is rich in fish liver (Hajimoradi et al., 2009). Hence, FW acclimation in ayu larvae could lead to the enhancement of lipid utilization. Aldolase B plays a key role in carbohydrate metabolism as it catalyzes one of the major steps of the glycolytic-gluconeogenic pathway (Munnich et al., 1985). This suggests that FW acclimation in ayu larvae might lead to the enrichment of carbohydrate metabolism. In addition, ATP synthase was also upregulated in FW-acclimated ayu larvae. Osmoregulation is a physiological process of energy consumption (Tseng & Hwang, 2008). Enhanced expression of ATP synthase may contribute to energy for osmoregulation. Hence, FW acclimation may enhance the utilization of amino acids and lipids to produce more ATP to regulate salinity change according to gene changes between FW- and BW-acclimated ayu larvae.

Direct osmoregulation in FW-acclimated ayu larvae included a variety of genes related to different physiological processes. Firstly, *BNP* was downregulated in FW-acclimated larvae. Natriuretic peptides are in charge of blood volume and osmosis (Loretz & Pollina, 2000; Martinez-Rumayor et al., 2008). In fish, the primary actions of natriuretic peptides are extrusion of excess salt and limitation of drinking-coupled salt uptake (Loretz & Pollina, 2000). *BNP* was downregulated one week after FW acclimation. Hence, the downregulation of *BNP* in FW-acclimated larvae might be the direct mechanism of osmoregulation. NKA provides the primary driving force for ion concentration regulation after changes in environmental salinity (Gonzalez, 2012). The mRNA expression of *NKA* was upregulated in FW-acclimated larvae and might directly contribute to osmoregulation via maintaining ion balance. Moreover, we found that *BHMT*, an enzyme involved in a cell volume-regulatory response via regulating betaine (Lu et al., 2010), was upregulated in FW-acclimated larvae, suggesting that FW acclimation may also regulate cell volume in ayu larvae. We also found that *transgelin*, an actin-binding protein involved in protein synthesis and destination (Assinder et al., 2009), was downregulated in FW-acclimated larvae, indicating that many protein synthesis processes might be depressed in FW-acclimated ayu larvae. Furthermore, *ID* was upregulated in FW-acclimated ayu larvae. ID is a subfamily of deiodinase enzymes important in the activation and deactivation of thyroid hormones, which are believed to play an osmoregulatory role in freshwater teleosts (Subash Peter et al., 2000). This suggests that the upregulation of *ID* in FW-acclimated larvae might also directly contribute to osmoregulation via regulation of the thyroid hormones.

In this study, we found that ayu larval growth was suppressed in FW-acclimated larvae compared with that in BW-acclimated larvae. This might result from hormones regulating food intake, catabolism of amino acids and lipids in FW-acclimated ayu larvae, and increased consumption of ATP to maintain osmotic pressure.

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ZOOLOGICAL RESEARCH

Expression analysis of eight amphioxus genes involved in the Wnt/β-catenin signaling pathway

Jing WANG[1,#], Guang LI[1,2,#], Guang-Hui QIAN[1], Jun-Hao HUA[1], Yi-Quan WANG[1,2,*]

[1] *State Key Laboratory of Cellular Stress Biology*, *School of Life Sciences*, *Xiamen University*, *Xiamen* 361102, *China*

[2] *Shenzhen Research Institute of Xiamen University*, *Shenzhen* 518057, *China*

ABSTRACT

The Wnt/β-catenin signaling pathway plays a crucial role in the embryonic development of metazoans. Although the pathway has been studied extensively in many model animals, its function in amphioxus, the most primitive chordate, remains largely uncharacterized. To obtain basic data for functional analysis, we identified and isolated seven genes (*Lrp5/6*, *Dvl*, *APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro*) of the Wnt/β-catenin signaling pathway from the amphioxus (*Branchiostoma floridae*) genome. Phylogenetic analysis revealed that amphioxus had fewer members of each gene family than that found in vertebrates. Whole-mount *in situ* hybridization showed that the genes were maternally expressed and broadly distributed throughout the whole embryo at the cleavage and blastula stages. Among them, *Dvl* was expressed asymmetrically towards the animal pole, while the others were evenly distributed in all blastomeres. At the mid-gastrula stage, the genes were specifically expressed in the primitive endomesoderm, but displayed different patterns. When the embryo developed into the neurula stage, the gene expressions were mainly detected in either paraxial somites or the tail bud. With the development of the embryo, the expression levels further decreased gradually and remained only in some pharyngeal regions or the tail bud at the larva stage. Our results suggest that the Wnt/β-catenin pathway might be involved in amphioxus somite formation and posterior growth, but not in endomesoderm specification.

Keywords: Wnt/β-catenin signaling pathway; Gene expression; Amphioxus; Whole-mount *in situ* hybridization; Embryo

INTRODUCTION

The Wnt/β-catenin signaling pathway acts as a major route passing signals from the outside to the inside of a cell. The signaling is initiated by binding of Wnt ligands to Frizzled receptors and Lrp5/6 co-receptors, which in turn results in recruitment of Axin and Dvl to the cell membrane and succedent disassembly of β-catenin from the Apc-Axin-Gsk3β-CkI complex. After that, the released β-catenin proteins translocate to the nucleus and interact with the Tcf/Lef transcription factor to activate target gene expression (Logan & Nusse, 2004; Rao & Kuhl, 2008). Comparison among available genome sequences shows that members of this pathway are conserved throughout all metazoan clades, but not among fungi, plants, or unicellular eukaryotes (Holstein, 2012).

The conservation of the Wnt/β-catenin signaling pathway indicates its important role in metazoic embryogenesis. Indeed, functional studies have demonstrated that the signaling controls many aspects of metazoic development, including germ layer specification, axis patterning, and posterior growth (Hikasa & Sokol, 2013; Martin & Kimelman, 2009; Petersen & Reddien, 2009). In vertebrates, the signaling plays an early role in dorsal-ventral (D-V) axis determination and a late role in anterior-posterior (A-P) axis development and posterior growth regulation (Hikasa & Sokol, 2013; Martin & Kimelman, 2009). In invertebrate deuterostomes, such as *Ciona*, sea urchins, and hemichordates, the signaling is essential for both endomesoderm specification and A-P axis development (Darras et al., 2011; Imai et al., 2000; Logan et al., 1999; McCauley et al., 2015; Momose et al., 2008; Momose & Houliston, 2007; Wikramanayake et al., 1998, 2003). In protostomes, the function of this signaling pathway exhibits considerable divergence among taxonomic groups. For example, in *Caenorhabditis elegans* and *Drosophila melanogaster*, the

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*Corresponding author, E-mail: wangyq@xmu.ecu.cn

#Authors contributed equally to this work

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signaling pathway regulates cell asymmetric division (Mizumoto & Sawa, 2007), but in nemertean *Cerebratulus lacteus* it participates in endoderm and A-P axis formation (Henry et al., 2008) and in short-germ band insects and spiders, it plays an essential role in posterior growth.

It appears that the function of the Wnt/β-catenin signaling pathway evolved distinctively during metazoan evolution, but questions about this evolvement have not yet been fully addressed. To increase our understanding of this pathway, we analyzed the expression profiles of eight genes involved in Wnt signaling at various developmental stages in amphioxus embryos. Amphioxus occupies a key position in the phylogeny of chordates (Delsuc et al., 2006), and its embryogenesis shows similarities to that of invertebrates (before the gastrula stage) and vertebrates (after the neurula stage). Importantly, amphioxus embryos have a clear A-P axis (Holland & Holland, 2007) and a posterior growth zone (tail bud) (Schubert et al., 2001), making them ideal research models to clarify the above questions. Previous studies have indicated that Wnt signaling is likely involved in amphioxus A-P axis determination, but not in D-V specification (Holland et al., 2005; Onai et al., 2009, 2012). However, whether signaling regulates amphioxus endomesoderm specification and posterior growth still remains to be elucidated.

MATERIALS AND METHODS

Animals and embryos

Originally provided from Jr-Kai Yu's lab by Dr. Zhang in 2011, amphioxus *Branchiostoma floridae* was introduced to our lab and cultured as described previously for *B. belcheri* (Li et al., 2012, 2013). Mature males and females with well-developed gonads were induced to spawn via thermal shock (Li et al., 2013). The eggs were fertilized *in vitro* and raised in dishes. Embryos at different developmental stages, including one-cell, two-cell, four-cell, blastula, mid-gastrula, early neurula (neural plate), mid-neurula (5-6 somites), late neurula (10-15 somites), and early larva (mouth open), were collected and fixed in 4% paraformaldehyde (PFA) dissolved in 4-morpholinepropanesulfonic acid (MOPS) buffer for whole-mount *in situ* hybridization (WISH).

Gene identification and cDNA cloning

Sequences of amphioxus *Lrp5/6*, *Dvl*, *APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro* genes were retrieved from the NCBI database using the Tblastn program with mouse homologous sequences as queries, and further verified with our transcriptome dataset. Primer pairs for each target gene were then designed (Table 1) and used to amplify the gene fragments using cDNA templates derived from gastrulae and neurulae. The amplified fragments were separately purified using a gel extraction kit (Omega, USA), subcloned into pGEM-T easy vector (Promega, USA), and verified by sequencing analysis.

Phylogenetic analysis

For each target gene, homologous sequences from *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Ciona intestinalis*, *Saccoglossus kowalevskii*, and *Strongylocentrotus purpuratus* were downloaded from public databases and aligned using the ClustalW program in MEGA5 software (Tamura et al., 2011). The alignment files were then used to construct neighbor-joining (NJ) trees using MEGA5 software with the Poisson model and 500 bootstrap replications.

Table 1 Primer sequences used for cDNA cloning and vector-construction

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Lrp5/6	GACCGATGCCTAGCCAAGT	CTTCTCGCCAGCAGGAGGATT
Dvl	ATGGAGGAGACGAAAATCATT	TCACATGACGTCCACGAAG
Axin	ATGAGTATTGGTGTACAGGAATAC[0]	TCACTCTATGCGCTCCACCTT
Apc	AGCTCGGTTGCTACAGGAGATAG	GTGCCGACAAGTTCCACAAA
CkIα	GGTACCCTTTGGGACAGAATGGCGAGC	ACTAGTCACCTCAGCGTCCTTTAC
CkIδ	AGAGCCGAGATGGAGTTGA	CTCCGACTACTTCTTGATG
Gsk3β	ATGAGCGGAAGACCACGGACAAC	TTATGTCCCCCCAGAGGCTGATGCG
Gro	ATGTATCCTCAAAACAGAC	TCAGTAGATGACTTCGTAAAG

Whole-mount *in situ* hybridization

Digoxigenin-labeled (Roche, USA) sense and antisense probes for target genes were synthesized using Sp6 or T7 RNA polymerase from the templates of linearized recombination pGEM-T easy vectors, and WISH was performed according to previously reported methods (Yu & Holland, 2009), with slight modification. Before the WISH experiments, envelopes of unhatched embryos were removed with a slim glass pin to facilitate reagent penetration. After proper staining, the embryos were fully washed with PBS, and were then mounted in 80% glycerol and photographed under an inverted microscope.

RESULTS

Identification of Wnt/β-catenin signaling components in amphioxus

Several genes encoding the components in the Wnt/β-catenin signaling pathway have been described in amphioxus in previous research (Holland, 2002; Lin et al., 2006; Qian et al., 2013; Yu et al., 2007). In the present study, we isolated another seven genes, including *Lrp5/6*, *Dvl, APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro*, from the genome of *Branchiostoma floridae*. Results

indicated that amphioxus possesses all genes involved in the Wnt/β-catenin signaling pathway, but fewer homologous genes than that observed in vertebrates (Supplementary Figure 1-7 and Supplementary Table 1). For example, two Wnt co-receptor genes (*Lrp5* and *Lrp6*) exist in the vertebrate genome, but a single ortholog (*Lrp5/6*) was found in the amphioxus genome; three *Dvl* genes exist in the genomes of most vertebrate species, but only one counterpart was detected in amphioxus. These observations are consistent with the hypothesis of a pre-duplicated genome status in amphioxus, indicating that components of the Wnt/β-catenin signaling pathway have not experienced lineage-specific expansion and should be much simpler than that in vertebrates.

Expression pattern of genes involved in the Wnt/β-catenin signaling pathway

To investigate the function of the Wnt/β-catenin signaling pathway during amphioxus embryogenesis, we further determined the expression patterns of the seven newly identified genes using the WISH method. The *Axin* gene was included in the present analysis since its expression at early development stages has not been determined in previous studies (Beaster-Jones et al., 2008). Figures 1, 2, and 3 are overviews of the gene expression patterns. From the one-cell to late blastula stages, the transcripts of *Lrp5/6*, *Axin*, *APC*, *Ckl*α, *Ckl*δ, *Gsk3β*, and *Gro* appeared evenly throughout the developing embryos, but that of *Dvl* was asymmetrically localized towards the animal pole (Figure 1, column A to D), which was denoted by the *Vasa* gene expression at the opposite vegetal pole (Figure 1A2, B2). At the gastrula stage, the expressions of the genes became undetectable in the ectoderm and conspicuously appeared in the primitive endomesoderm (Figure 1, column E and F). Interestingly, these genes were differentially expressed in the endomesoderm, with *Lrp5/6* mainly expressed in the dorsal and lateral regions, *Dvl* and *Gro* primarily expressed in the dorsal-anterior region, *APC* and two *Ckl*s expressed in the dorsal region, *Axin* expressed in the blastopore rim, and *Gsk3β* evenly expressed throughout the endomesoderm. These patterns were typically maintained until the early neurula stage, except that the signal in the dorsal mesoderm became more conspicuous than that in other tissue (Figure 2, column A to C). At the mid and late neurula stages, the expressions of these genes were commonly detected in the paraxial mesoderm (somites) (Figure 2, column D and E; Figure 3, column A and B). Other expression sites were also detected for the *Axin* gene in the tail bud, *Dvl* and *Gro* in the anterior endoderm diverticulum, and *Gsk3β* in the gut. At the larva stage, the expressions of the genes decreased and weak signals remained in some pharyngeal regions or the tail bud only (Figure 3, column C). We also synthesized sense probes of *Gsk3β*, *Gro*, and *APC* genes and performed side-by-side *in situ* hybridization experiments using both sense and antisense probes. No signal was observed in the embryos hybridized with the sense probes, but a strong specific staining was observed in the embryos hybridized with the antisense probes (Supplementary Figure 8).

DISCUSSION

Function of the Wnt/β-catenin signaling pathway in amphioxus early embryogenesis

The activation of Wnt/β-catenin signaling on one side of a cleavage-stage embryo is a crucial process in metazoan embryonic axis patterning and germ layer specification. In animals such as sea urchins (Logan et al., 1999), hemichordates (Darras et al., 2011), and *Ciona* (Imai et al., 2000), early regionalized Wnt signaling is localized to the vegetal pole and associated with endomesoderm specification. In cnidarians, the signaling is involved in endomesoderm specification (Martindale, 2005), although it is activated in the animal pole (Wikramanayake et al., 2003). In vertebrates, however, the signaling activity is enriched in the dorsal region, where it is crucial for Spemann-Mangold organizer formation and subsequent D-V axis determination (Hikasa & Sokol, 2013; Niehrs, 2004). This early regionalized Wnt signaling center is generally activated by localized expression of upstream signaling components such as Wnt ligands (Tao et al., 2005) and/or Dvl (Miller et al., 1999; Weitzel et al., 2004; Tadjuidje et al., 2011). To date, ten Wnt genes have been identified in amphioxus and eight of their developmental expression patterns have been analyzed by *in situ* hybridization; however, none of these genes have shown a detectable expression before the gastrula stage (Holland, 2002). Using RT-qPCR, five *Wnt* genes, including *Wnt1*, *6*, *9*, *10*, and *11*, were found to be maternally expressed, although expression levels were relatively low (Qian et al., 2013). However, it is not clear whether these genes are expressed asymmetrically at one pole of cleavage-stage embryos. In the present study, the amphioxus *Dvl* gene was asymmetrically expressed toward the animal pole in the amphioxus embryo from the one-cell to late blastula stages. This expression pattern is the same as that of amphibian *Dvl* (Miller et al., 1999; Tadjuidje et al., 2011), but different from that of sea urchin *Dvl*, which is enriched in the vegetal pole (Weitzel et al., 2004). Thus, it appears unlikely that amphioxus embryos could form a regionalized Wnt signaling center at the early stages via the asymmetric expression of the *Dvl* gene since the downstream effector β-catenin can localize nuclei of all embryonic blastomeres from the 16-cell to late blastula stages (Holland et al., 2005). This possibility is further strengthened by the ubiquitous expression of *Lrp5/6*, *Axin*, *APC*, *Ckl*α, *Ckl*δ, *Gsk3β*, and *Gro* (present study) and four *Frizzled* genes (Qian et al., 2013), and the undetectable expression of Wnt/β-catenin signaling antagonists such as *Dkk*s, *sFrp*s, and *Ceberus* in amphioxus embryos before the gastrula stage (Yu et al., 2007; Onai et al., 2010, 2012; unpublished data). Results suggest that Wnt signaling is probably not involved in endomesoderm specification or D-V axis determination in amphioxus (Holland et al., 2005). Consistent with this assumption, the upregulation of Wnt signaling through the inhibition of Gsk3β activity by lithium chloride before the mid-blastula stage has no effect on mesendoderm specification or D-V axis development (Holland et al., 2005). The evidence supporting amphioxus having a regionalized Wnt/β-catenin signaling center in the cleavage-stage embryo is the

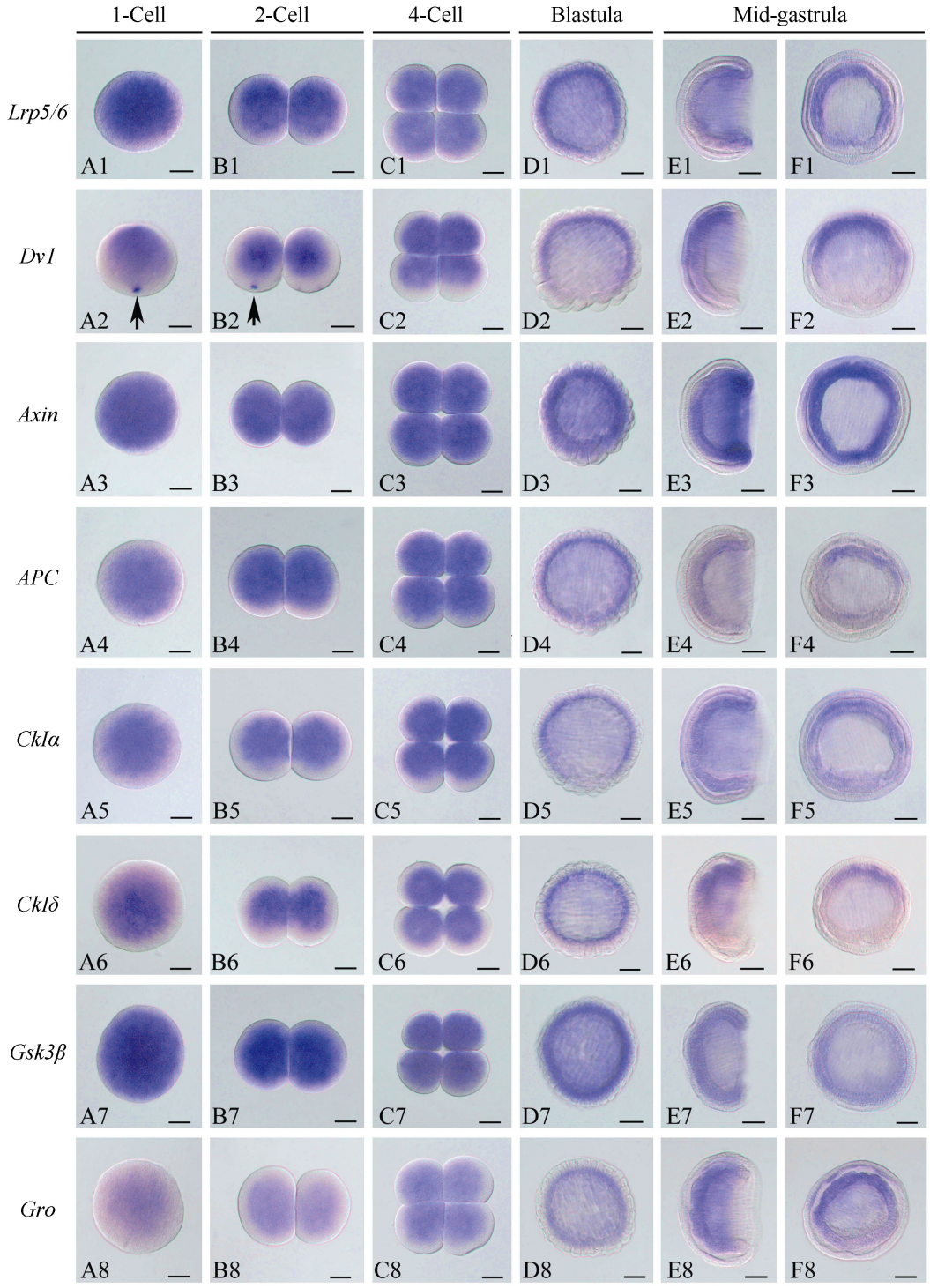

Figure 1 Overview of the expression patterns of eight Wnt/β-catenin signaling genes in one-cell to mid-gastrula stages of amphioxus embryos
Embryos in columns A, B, D, and E are in side view, embryos in column C are in animal-vegetal axis view, and those in column F are in blastopore view. Embryos in A2 and B2 were hybridized with *Dvl* and *Vasa* (vegetal marker) probes simultaneously to show the asymmetrical expression of *Dvl* in the animal pole. Scale bars=50 μm.

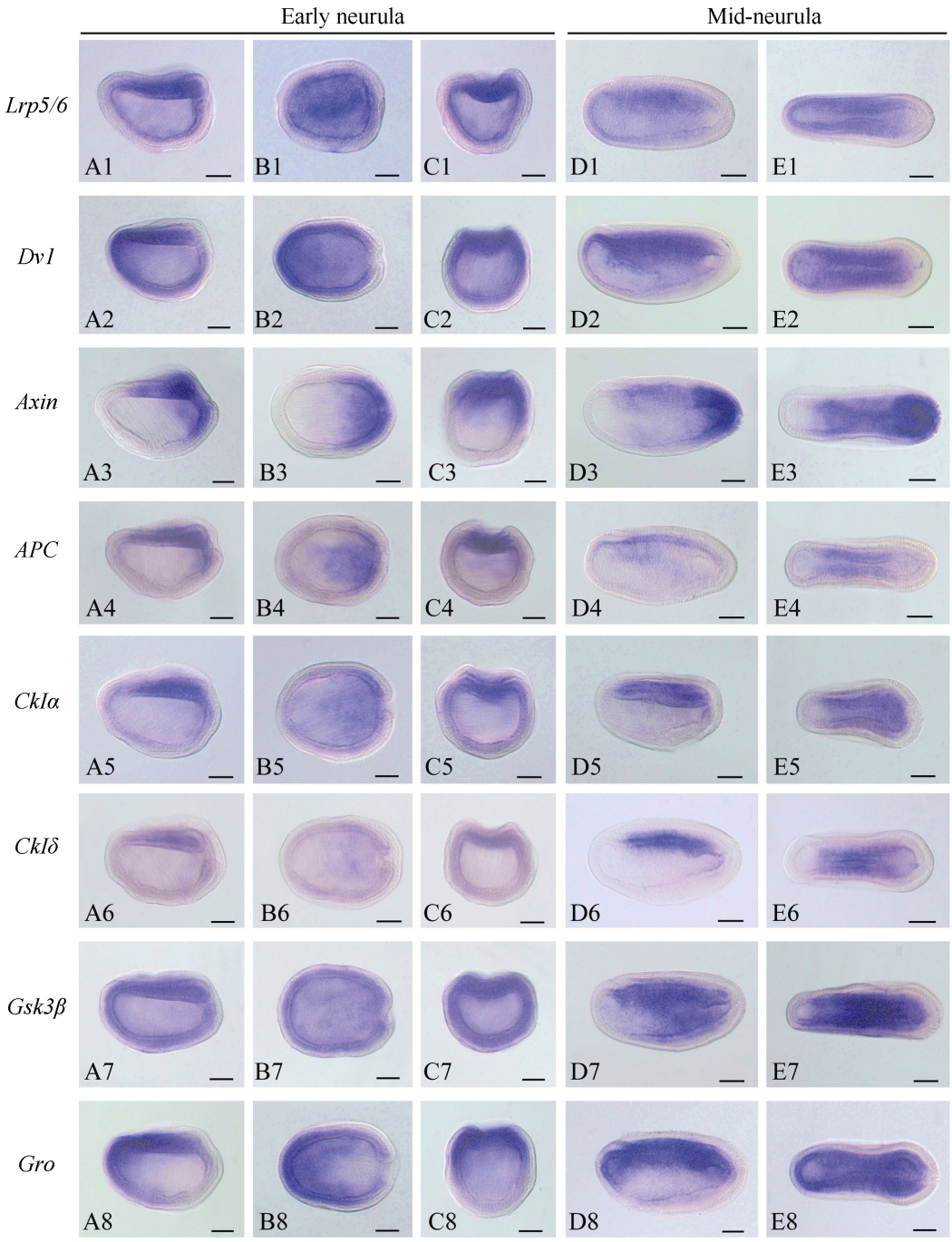

Figure 2 Overview of the expression patterns of eight Wnt/β-catenin signaling genes in early and mid-neurula stages of amphioxus embryos

Embryos in columns A and D are in side view, embryos in column C are in blastopore view, and those in columns B and E are in dorsal view. Scale bars=50 μm.

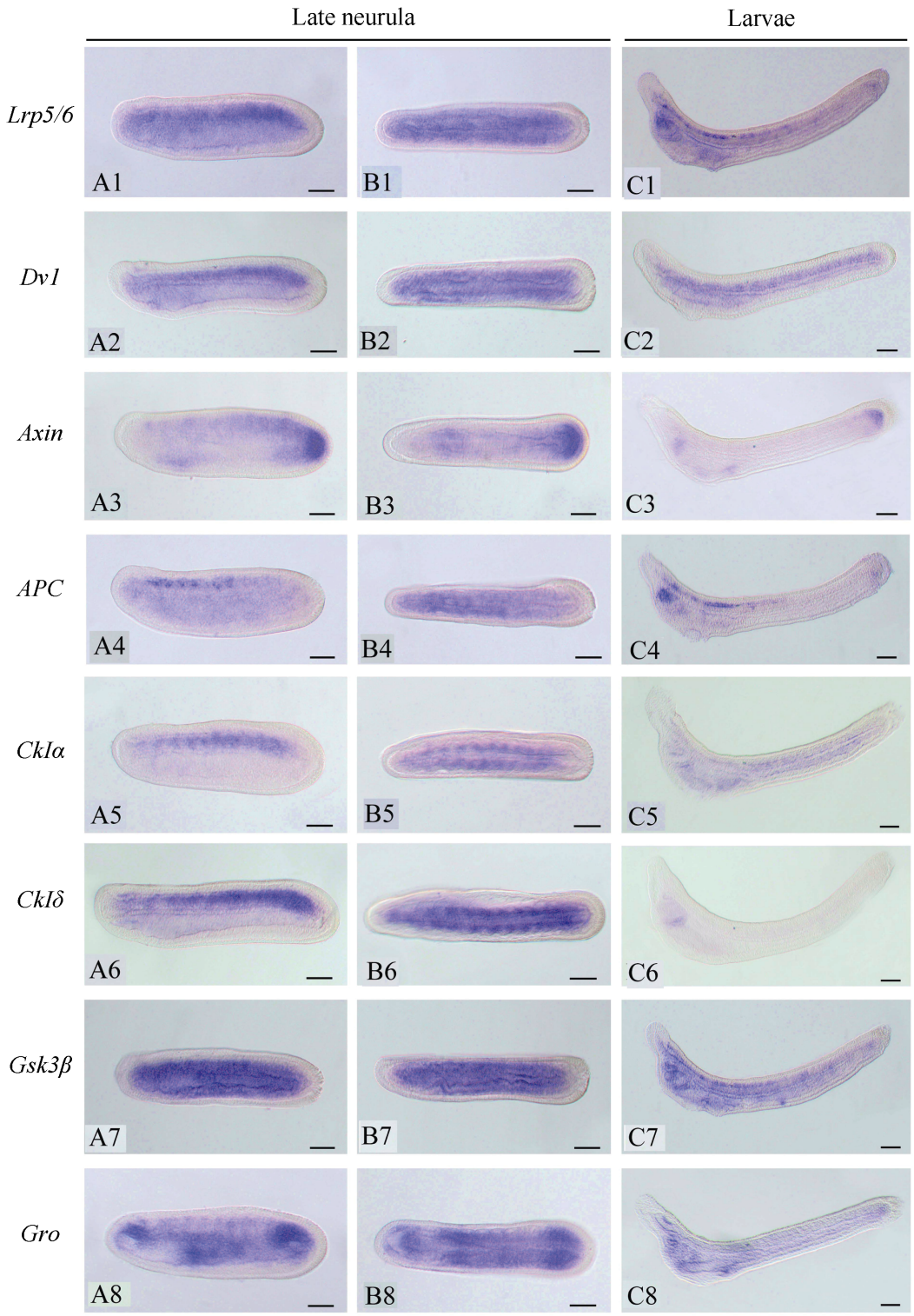

Figure 3 Overview of the expression patterns of eight Wnt/β-catenin signaling genes at late neurula and early larva stages
Figures in columns A and C are in side view and those in column B are in dorsal view. Scale bars=50 μm.

asymmetrical expression of the *Tcf* gene in the animal pole of early stage embryos (Lin et al., 2006). It would be interesting to perform functional study on the amphioxus *Tcf* gene via over-expression and knockdown experiments in the future.

Function of Wnt/β-catenin signaling in amphioxus posterior growth and somitogenesis

Body elongation via posterior growth is an important feature during the late stage of vertebrate embryogenesis. This elongation is accomplished by means of continuously adding segmented somite blocks to the posterior region (tail bud) at the neurula stage. Wnt signaling plays an essential role during the process by coordinating mesoderm formation and segmentation (Dunty et al., 2008). Vertebrate embryos lacking Wnt signaling components like Wnt3a or β-catenin display posterior axis truncations and form only head structures and anterior parts of the trunk (Dunty et al., 2008; Takada et al., 1994). Posterior growth does not exist in laboratory non-invertebrate metazoan animals such as sea urchins, *Ciona*, *Drosophila*, and *Caenorhabditis elegans*. This makes determining whether Wnt signaling regulates posterior growth in animals outside of vertebrates elusive. However, several studies on short germ-band insects, including the red flour beetle and cricket, have indicated a conserved role for Wnt signaling in posterior growth (Bolognesi et al., 2008; Miyawaki et al., 2004), although the genetic network underlying it is relatively different between insects and vertebrates (Martin & Kimelman, 2009). Amphioxus embryos have a posterior growth zone (tail bud) from which posterior somites can continuously bud off in a one-left-one-right manner (Schubert et al., 2001). Based on the expressions of *Wnt* and target genes, such as *Brachyury* and *Caudal*, within or around the tail bud of amphioxus embryos, Holland (2002) speculated that the Wnt signaling pathway should play a critical role in amphioxus posterior growth. Studies on other amphioxus Wnt signaling components, including *β-catenin* (Holland et al., 2005), *Tcf* (Lin et al., 2006), *Frizzled*s (Qian et al., 2013), *Lrp5/6*, *Dvl*, *Axin*, *Ck*s, *Gsk3β*, and *Gro* (present study), have further strengthened this speculation as these genes were transcribed within or around the amphioxus tail bud region. These results have also implicated that posterior growth mediated by Wnt/β-catenin signaling might be an ancient characteristic in metazoans and the genetic toolkit underlying this process would be relatively complete in amphioxus.

The Wnt/β-catenin signaling pathway is also essential for early stages of somitogenesis in vertebrates by maintaining the expression of myogenesis gene *MyoD* and inducing axial mesoderm inhibitor genes *Vent*/*Vox* (Hoppler & Moon, 1998; Ramel & Lekven, 2004). However, after the commitment of paraxial mesoderm to somatic fate, Wnt signaling is rapidly downregulated and not required for late myogenesis (Tian et al., 1999). Preliminary analysis on *Wnt8* expression patterns indicated a similar role for Wnt signaling in amphioxus early somitogenesis (Schubert et al., 2000). It also revealed that amphioxus *Wnt8* was continuously transcribed in some of the differentiated somites, indicating an essential role for Wnt/β-catenin signaling in both early stage somite formation and somite differentiation (Schubert et al., 2000). Consistent with this observation, we found that amphioxus *Lrp5/6*, *Dvl*, *Axin*, *APC*, *Ck*s *Gsk3β*, and *Gro* were all expressed in most differentiated somites in the mid to late neurulae. Further functional experiments are necessary to clarify this hypothesis.

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Expression levels of *GSTA2* and *APOD* genes might be associated with carotenoid coloration in golden pheasant (*Chrysolophus pictus*) plumage

Guang-Qi GAO, Li-Shuang SONG, Bin TONG, Guang-Peng LI*

Research Center for Laboratory of Animal Science, *Inner Mongolia University, Hohhot* 010070, *China*

ABSTRACT

Carotenoids, which generate yellow, orange, and red colors, are crucial pigments in avian plumage. Investigations into genes associated with carotenoid-based coloration in avian species are important; however, such research is difficult because carotenoids cannot be synthetized in vertebrates as they are only derived from dietary sources. Here, the golden pheasant (*Chrysolophus pictus*) was used as a model in analysis of candidate gene expression profiles implicated in carotenoid binding and deposition. Using mass and Raman spectrometry to confirm the presence of carotenoids in golden pheasant feathers, we found $C_{40}H_{54}O$ and $C_{40}H_{56}O_2$ in feathers with yellow to red colors, and in the rachis of iridescent feathers. The global gene expression profiles in golden pheasant skins were analyzed by RNA-seq and all six carotenoid binding candidate genes sequenced were studied by real-time PCR. *StAR*4, *GSTA*2, *Scarb*1, and *APOD* in feather follicles showed different expressions in red breast and orange nape feathers compared with that of iridescent mantle feathers. Further comparison of golden pheasant yellow rump and Lady Amherst's pheasant (*Chrysolophus amherstiae*) white nape feathers suggested that *GSTA*2 and *APOD* played a potential role in carotenoid-based coloration in golden pheasant.

Keywords: Expression; Carotenoid coloration; Candidate genes; Golden pheasant; Feather

INTRODUCTION

Golden pheasant (*Chrysolophus pictus*) is a world-renowned avian species with particularly attractive and varied plumage. As one of the most prevalent integument pigments in birds, carotenoids are usually deposited in bare body parts and produce yellow, orange, and red coloration in feathers (usually in the distal parts). To date, around 30 different carotenoids have been identified in hundreds of avian species (Hill & McGraw, 2006; LaFountain et al., 2010). Furthermore, this class of pigment has been confirmed in 95 of 236 extant bird families, including three species of Galliformes, such as the golden pheasant (Thomas et al., 2014a). Unlike other pigments, such as melanin, carotenoids are difficult to study in regards to the genetics of bird coloration because knowledge of the molecular genetics of carotenoid-based coloration is incomplete (Roulin & Ducrest, 2013). Under this limitation, transportation, deposition, and metabolism-related proteins and their encoding genes have been studied in consideration of the dietary-derived characteristics of these pigments (Pointer et al., 2012; Walsh et al., 2012). In chicken skins, mutations of beta-carotene dioxygenase 2 (BCDO2) inhibit gene expression and result in carotenoid accumulation (Eriksson et al., 2008). In bird feathers, carotenoids are bound directly to keratin or other proteins (Stradi et al., 1995; Mendes-Pinto et al., 2012), so it is generally accepted that carotenoid-binding protein (CBP) is the starting point to investigate carotenoid-based coloration of plumage at the molecular level.

A previous report reviewed 11 plausible candidate genes associated with carotenoid uptake, binding, and deposition, including steroidogenic acute regulatory protein 4 (*StAR*4), glutathione S-transferase alpha 2 (*GSTA*2), StAR-related lipid transfer domain containing 3 (*STARD*3, also known as *MLN*64), scavenger receptor class B, member 1 (*Scarb*1, also known as *SR-BI*), apolipoprotein D (*APOD*), and lipid storage droplet 2-like (*PLIN*) genes (Walsh et al., 2012). As a member of the StAR protein family, StAR4 specifically binds lutein with high affinity, and is a possible candidate for CBP in human retina (Bhosale et al., 2009). Scarb1 is involved in carotenoid

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*Corresponding author, E-mail: gpengli@imu.edu.cn

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transport into retinal pigment epithelium (RPE) cells, and gene silencing can result in a marked inhibition of carotenoid uptake (During et al., 2008). Although *APOD* is a carotenoid-binding gene in crustaceans and mammals, a paralogous gene has since been characterized in chickens, and is expressed in skins and developing feathers (Ganfornina et al., 2005). *GSTA*2 is the closest avian homologue of the carotenoid binding gene *GSTP*1 in mammalian retina and is involved in plumage dichromatism (Walsh et al., 2012; Zhang et al., 2014). It is predicted, therefore, that these genes might be highly expressed in carotenoid feathers.

Prior to pigment identification, we analyzed the expression levels of six carotenoid deposition genes in golden pheasant feather follicles, in which the corresponding feathers may or may not have contained carotenoids.

MATERIALS AND METHODS

Animals and samples

Three male artificially bred golden pheasants were used in this study. The birds were provided by the Yuanfeng Wildlife Farm (Jilin, China) and were housed in cages with free access to water and commercial food. This study was approved by the Institutional Animal Care and Use Committee of the Inner Mongolia University. For RNA extraction, skins and feather follicles were sampled. Briefly, feathers of various colors were plucked from the breast, nape, rump, and mantle of the body (Figure 1). Regrown feather shafts and skins were then collected and stored in liquid nitrogen (Poelstra et al., 2014). For pigment extraction, feathers were plucked from corresponding regions directly, then placed in envelopes and stored in the dark. Blood samples were drawn from the neck veins using vacuum blood collection tubes, with the samples then centrifuged in heparinized microcapillary tubes for 10 min at 1 500 *g*. The plasma was then removed and stored at –80 °C in 1.5 mL Eppendorf tubes until further analysis (McGraw et al., 2002a).

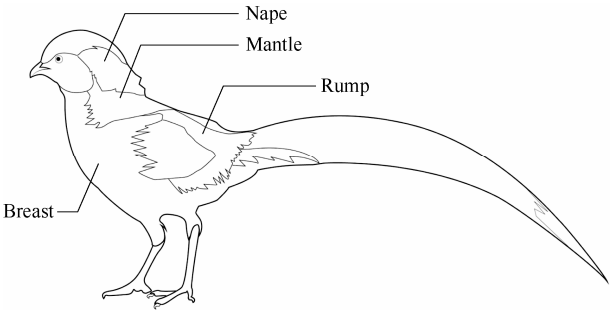

Figure 1 Feathers and feather follicles sampled from the breast, nape, mantle and rump of the golden pheasant

Feathers from breast, nape and rump were red, orange and yellow in the distal parts, while feathers from the mantle were iridescent green. Feather follicles were sampled during the growing period.

RNA-seq

The isolated mRNA from golden pheasant skins was fragmented and used for cDNA synthesis. The short cDNA fragments were treated following the manufacturer's instructions and sequenced using an Illumina Hiseq 2000 (Illumina, San Diego, USA). The produced reads were assembled by mapping to the golden pheasant genome we sequenced and annotated based on homology prediction. The expression abundance of each assembled transcript was measured through reads per kilobase transcriptome per million mapped reads (RPKM) (Mortazavi et al., 2008). The expressions of each read between sample pairs were calculated according to RPKM values in different samples. A minimum two-fold difference was considered as an expression difference with a false discovery rate (FDR) ≤0.05 (Benjamini et al., 2001). GO enrichment analysis was performed using GO: TermFinder software (http: //smd.stanford.edu/help/ GO-TermFinder/GO_TermFinder_help.shtml/).

Carotenoids extraction

Feather and plasma carotenoid pigments were extracted, respectively, using previously published methods (McGraw et al., 2002b, 2005). In brief, feathers from various parts of the body were soaked in ethanol and hexane, in turn, in order to remove surface lipids. Feather vanes were trimmed into pieces and placed into 10 mL glass tubes. After that, 1 mL of acidified pyridine was infused to cover the pieces. The tubes were then filled with argon gas, capped tightly, and placed in a 95 °C water bath for 3 h until the solution became colorful. After the thermochemical procedures, the cooled tubes were added with 2 mL of pure water and 1 mL of hexane: *tert*-butyl methyl ether (1:1) to separate the carotenoid pigments from the solution. The mixtures were shaken strongly for 2 min and centrifuged at 3000 r/min for 5 min, with the supernatants then transferred to clean glass tubes, dried under nitrogen gas, and stored at –80 °C until use. For blood samples, the extraction procedures were the same as above (McGraw et al., 2002a). Ten microliters of plasma with 100 μL of ethanol were mixed in 1.5 mL Eppendorf tubes, followed by the addition of 100 μL of *tert*-butyl methyl ether. After being vortexed, the tubes were centrifuged at 3 000 r/min for 3 min, with the same treatment as that for plumage pigment extraction.

Mass spectrometry

The extracted pigments were used for molecular weight analysis using an HPLC-MS instrument composed of a LC-20AD system (Shimadzu, Japan) fitted with a Waters™ C18 column (5 μm, 150 mm×2.1 mm) and an LTQ Velos Orbitrap (Thermo, Germany). Samples were dissolved in methanol and injected into the instrument at 0.2 mL/min and analyzed in the positive mode with the capillary heated to 350 °C and at a cone voltage of 4.5 kV. A gradient solvent system was used for compound elution, with the percentage of acetonitrile varying from 5% to 95% corresponding to the methanoic acid solution (v=5%) in the first 11 min, before decreasing to 5% in the next 3 min, and finally being held isocratically for 1 min.

Raman spectroscopy

Feather samples were examined in a Labram HR1800 spectrometer (HORIBA Jobin Yvon, France) without pretreatment using an excitation laser source at 514 nm and 0.3 mW power. Single spectra were obtained using a 100× confocal objective and a grating of 600 lines/mm. Data were analyzed by LabSpec 5 software. Standards of lutein (Chromadex, USA) and zeaxanthin (Sigma, USA) were used in comparative analysis.

Real-time PCR

Sequences of candidate genes were obtained from the RNA-seq data. The predicted sequences were used to design primers on exon regions spanning introns using Primer Premier 5 (Table 1). Housekeeping gene β-actin was used for normalization. Total RNA was extracted from the feather follicle samples taken from each of the three birds using RNAiso Plus reagent (Takara, Japan). cDNA preparation with quantitative real-time PCR for each candidate gene was performed using a PrimeScript^{TM} RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan). Each sample was pooled with 5-10 feather follicles. Reactions were performed in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA) with default parameters. Three replicates were used for each sample.

Table 1 Primers used for real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	TM (°C)
*StAR*4	CAAGCTATGAAGATGGGCTTC	ATCTGATTGCAAGGAATGCAC	60
*GSTA*2	TCCCTTTTCAAGCAGCCGAT	GCTGCCAGGCTGCAAGAAT	60
*STARD*3	GACCACGCACAGCCTGAA	AGGCTCTGGTGGATGAGGTA	60
*Scarb*1	ACTTCTACAATGCTGACCCAA	AGCTTGATGGAGCAATTCAT	60
APOD	GCGTCCGCTTCAACTGGT	CGTGGGCATCATCTTGTCG	60
PLIN	CCATCCAAAGTGCCAAGAG	CAGGTCTGCTTGGGCTTC	60
β-actin	CTCCCTGATGGTCAAGTCAT	TGGATACCACAGGACTCCAT	60

RESULTS

Carotenoid identification in golden pheasant plumage

Pigments were separated from golden pheasant plasma and feathers according to solubility. Both procedures result in carotenoid transfer into the upper organic phase and profile colors in it if this kind of pigment is present. We found yellow colors emerged in the mentioned phases after yellow rump, orange nape, and red breast feathers were treated. This indicated that yellow fat-soluble pigments were contained in these feathers, but not in the iridescent mantle feathers. The same phenomenon occurred in the plasma samples. To confirm the molecular weight of the compounds, the extracted pigments from plasma and plumage were analyzed by mass spectrometry. Search results in databases (METACYC, LIPID, KEGG) showed that $C_{40}H_{54}O$ and $C_{40}H_{56}O_2$ were present in all samples. According to previous publication, lutein and zeaxanthin are isomers with the formula $C_{40}H_{56}O_2$, and echinenone, which is a common carotenoid found in plumage, is $C_{40}H_{54}O$ (Hill & McGraw, 2006).

A single iridescent feather taken from the mantle region of the golden pheasant was examined by Raman spectroscopy for carotenoid identification, as described previously (Thomas et al., 2014a, b). We took two collection points at the rachis and barb, respectively, and found characteristic bands of carotenoids in the rachis at 1 500-1 535 /cm (identified as v[C=C]), 1 145-1 165 /cm (identified as v[C-C]), and 1 000-1 010 /cm (identified as δ [CH2]) (Thomas et al., 2014b). For the barb, bands of eumelanin (Galván et al., 2013) were detected (Figure 2).

Transcriptome sequencing and differential gene expression profiling

Four libraries were constructed for golden pheasant skin transcriptome study, including the breast, nape, mantle, and rump. Raw data were sequenced by an Illumina Hiseq 2000 and filtered to produce a total of 23.83 Gb clean reads. We mapped the reads to the golden pheasant genome to assemble the transcripts for each library. A summary of the mapping results is shown in Table 2. Transcriptome data were deposited in the Sequence Read Archive (SRA) database under accession no. SRP075618.

A total of 50 228 expressed genes were predicted. To find differences in gene expression between two kinds of color morphs, we compared the RPKM values of the transcripts in mantle skin to the other three parts. Results showed that 452, 748, and 532 genes were upregulated and 686, 1 185, and 710 genes were downregulated in the breast, nape and rump,

Table 2 Statistics of transcriptome sequencing data

	Breast	Nape	Mantle	Rump
Total reads	68 796 446	64 410 352	64 910 570	66 653 538
Total mapped reads	48 578 519	45 006 710	44 868 949	46 045 821
Total mapped rate (%)	70.61	69.87	69.12	69.08
Unique mapped reads	46 213 276	43 605 488	42 886 126	44 029 043
Unique mapped rate (%)	67.17	67.70	66.07	66.06

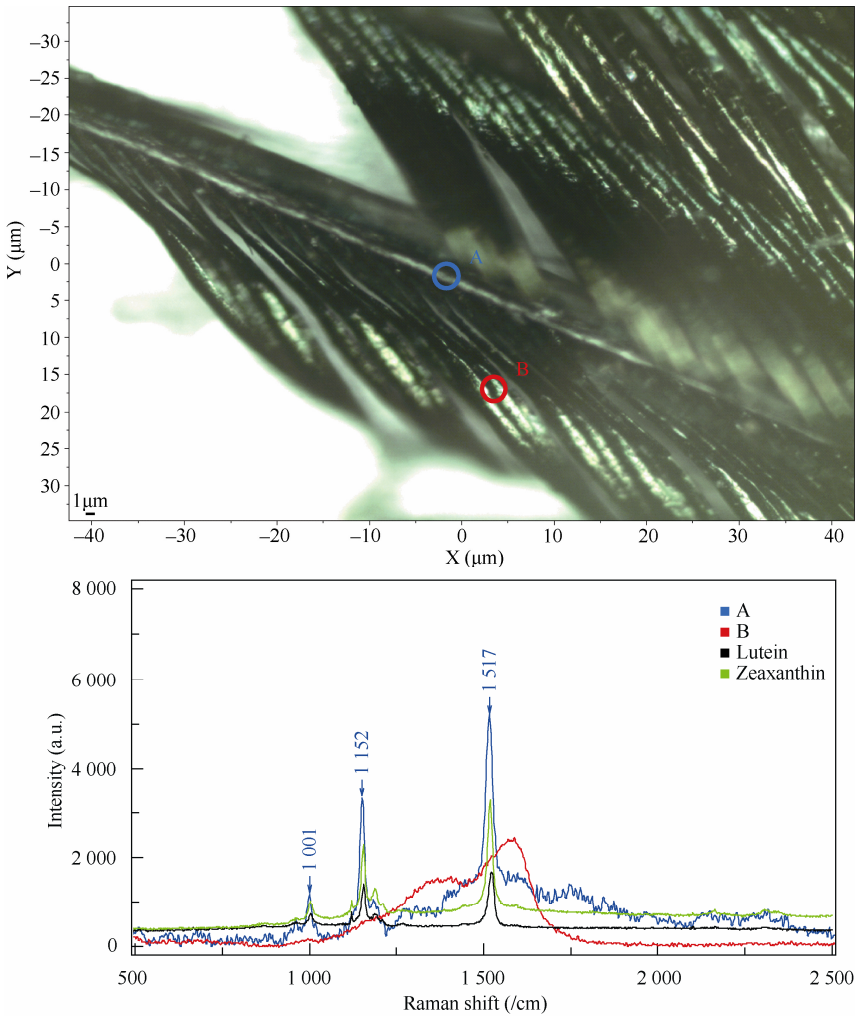

Figure 2 Detected points (above) and corresponding spectrograms (below) by Raman spectroscopy in an iridescent mantle feather of the golden pheasant

Purified standards of lutein and zeaxanthin were used as the positive controls.

respectively. The genes with significantly differential expression were enriched in 46 pathways, including the retinol metabolism pathway, which might be involved in carotenoid metabolism (Table S1). We enriched and overlapped the genes that showed preferential expression in carotenoid-containing tissues (Figure 3). Sixty-six genes were significantly expressed in all three carotenoid-containing tissues, of which 57 were functionally annotated (Table S2).

Expression of candidate genes in golden pheasant feather follicles

To confirm the expressions of carotenoid binding related genes in feather follicles, six homologous candidate genes were determined using real-time PCR. Expression differences in developing feather follicles taken from the rump, breast, nape, and mantle regions of the male golden pheasant were determined and the expression patterns were compared with RNA-seq data (Figure 4). All candidate genes were expressed in all tissue samples. Comparison of the expression patterns of the six genes revealed no differences (P>0.05) between yellow rump and iridescent mantle feather follicles. Compared with mantle tissue, there was no difference (P>0.05) in the expression levels of *STARD3* in the breast, nape and rump samples, and the expression of *PLIN* showed significant differences only in red breast feather follicles (P<0.05). The remaining five genes were differentially expressed in red and orange plumage. For red breast plumage, expressions of *StAR4* and *PLIN* showed significant differences (P<0.05), while expressions of *GSTA2*, *Scarb1*, and *APOD* showed highly significant differences (P<0.01). For orange nape plumage, expressions of *StAR4* and *APOD* showed significant differences (P<0.05), while expressions of *GSTA2* and *Scarb1* showed highly significant differences (P<0.01). We also compared the expression levels of the candidate genes between golden

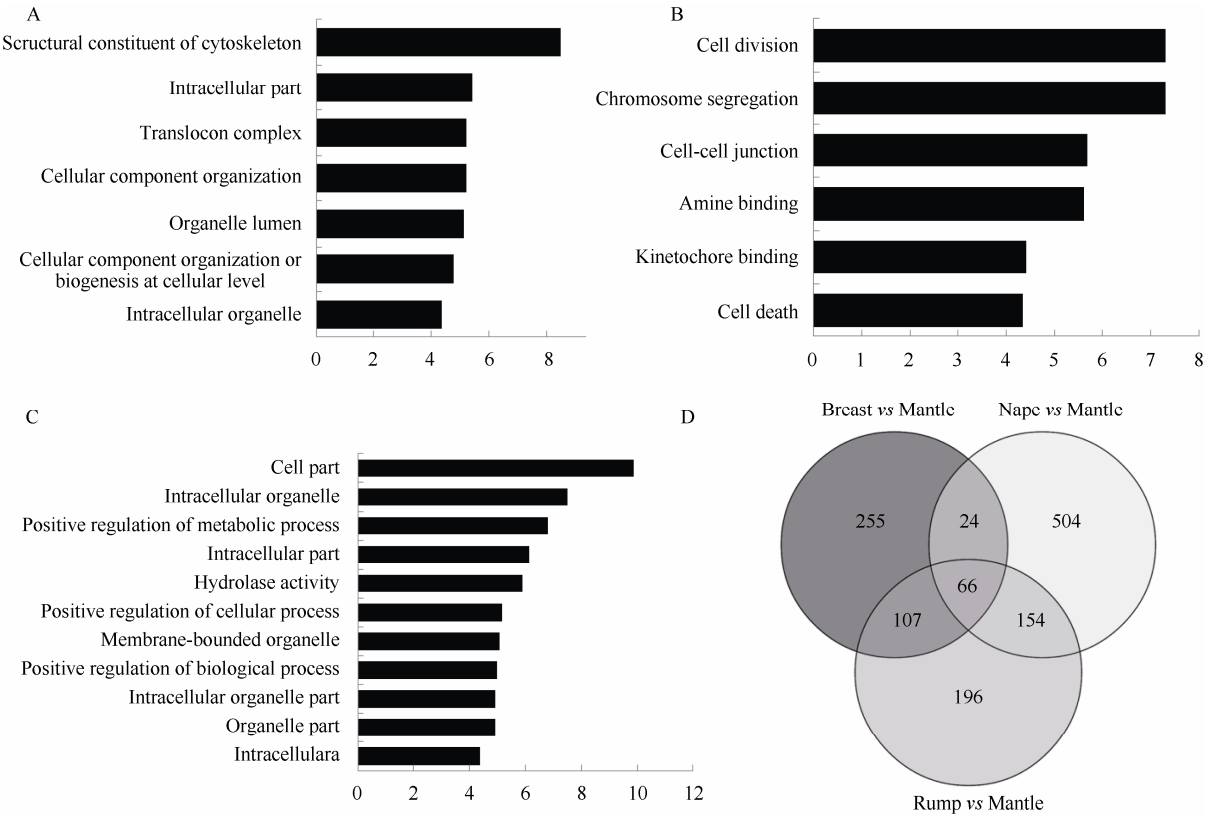

Figure 3 Analysis of differentially expressed genes (DEGs) upregulated in golden pheasant breast, nape, and rump compared with that in the mantle

A-C: GO enrichment of DEGs in breast (A), nape (B), and rump (C). X axes indicate -Log$_2$(*P*-value). D: Overlap of DEGs.

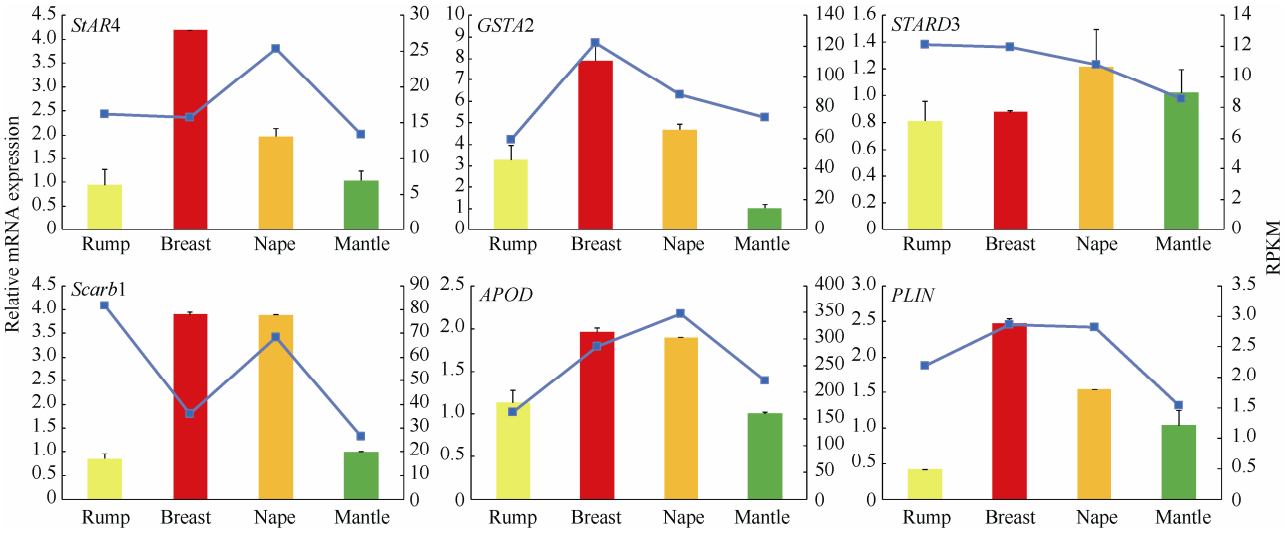

Figure 4 Expression levels of carotenoid candidate genes in different feather follicles of the golden pheasant (mean±*SD*) by real-time PCR

Columns indicate real-time PCR data, and lines indicate RNA-seq data. RPKM: Reads per kilobase transcriptome per million mapped reads.

pheasant yellow rump feathers and Lady Amherst's pheasantnape feathers, which are white with black stripes without carotenoid-based coloration. Data showed the expressions of *GSTA*2 and *APOD* were significantly different (Figure 5).

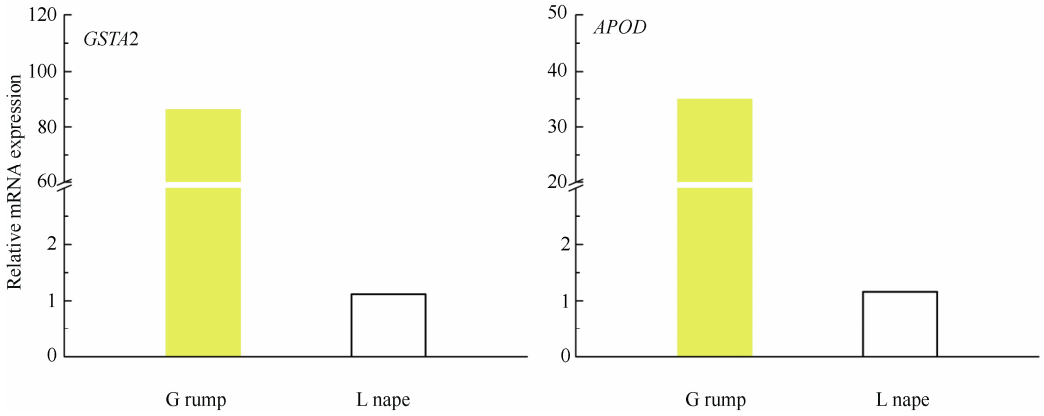

Figure 5 Expression levels using real-time PCR of carotenoid candidate genes in different feather follicles between golden pheasant (G) rump and Lady Amherst's pheasant (L) nape

DISCUSSION

Thermochemical techniques for carotenoid extraction are based on weakening the non-covalent hydrogen bonds that bind pigments to proteins, thereby subsequently releasing pigments into solution (McGraw et al., 2005). In our study on carotenoid separation from golden pheasant plumage, we found that the organic phases of red, orange, and yellow tested barbs were pigmented after treatment. This phenomenon suggested the presence of lipochrome in the feathers, with further mass spectrometry analysis confirming that these pigments were carotenoids. This conclusion was confirmed by searching molecular weights in databases (METACYC, LIPID, KEGG), with a focus on 40 specific carbon compounds. According to the common carotenoids documented in birds (Hill & McGraw, 2006), we suggest that the identified chemicals might be lutein, zeaxanthin, and echinenone. Therefore, it follows that at least two common dietary carotenoids (lutein and zeathanxin) exist in golden pheasant plumage.

As the plasma samples achieved the same results in carotenoid identification, we inferred that this kind of dietary-derived pigment was absorbed and transported into feather follicles. Consequently, we determined the expression of several carotenoid-binding genes in golden pheasant feather follicles and found some patterns were significantly different between carotenoid-containing samples and mantle samples, in which carotenoids were absent in barbs.

Genes related to carotenoid deposition did not show significant differential expression through RNA-seq analysis. Consequently, we performed real-time PCR and found that four genes (*StAR*4, *GSTA*2, *Scarb*1, and *APOD*) were highly expressed in red breast and orange nape feathers, suggesting their possible function in carotenoid deposition in these tissues of golden pheasant. The differences in expression indicate that these genes might play different roles in carotenoid coloration in different feathers. In addition, all candidate genes were expressed in all tissues, even iridescent mantle feather follicles, while, the expression levels in yellow rump feathers showed no differences compared with iridescent mantle feathers. To clarify this issue, we tested a mantle feather using Raman spectroscopy and found carotenoids in the rachis, but not in the barbs. This led to the hypothesis that the transported carotenoids might combine with some other factors in the mantle feather rachis, such as keratins (McGraw et al., 2003), and thus the expression of carotenoid-binding genes might not be the only reason for carotenoid deposition in these regions. Compared with the mantle feathers, *GSTA*2 and *APOD* were highly expressed in red breast and orange nape feathers, but not in yellow rump feathers in which carotenoids were detected. Therefore, a further comparison with white control feathers was carried out. Results indicated that the two genes might be important to carotenoid deposition in golden pheasant plumage.

The genes involved in carotenoid deposition in feathers are poorly known, yet are of substantial interest. The golden pheasant is an excellent species to address these questions, being one of the few Galliformes to express carotenoids in feather follicles. Six homologous candidate genes documented and investigated in *Quelea quelea* (Walsh et al., 2012) were studied in the golden pheasant in the present study. We found at least two candidate genes associated with carotenoid coloration, with differences in expressions raising the possibility that these genes might play different roles in golden pheasant plumage. Whether functional features of potential genes are species specific in golden pheasant or universal across avian taxa will spur the exploration of genetic and genomic investigations in future work.

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Effects of forest fragmentation on nocturnal Asian birds: A case study from Xishuangbanna, China

Salindra K. DAYANANDA[1,2,3,4], Eben GOODALE[2,*], Myung-bok LEE[2], Jia-Jia LIU[5], Christos MAMMIDES[6], Bonifacio O. PASION[1,4], Rui-Chang QUAN[1,*], J. W. Ferry SLIK[7], Rachakonda SREEKAR[1,8], Kyle W. TOMLINSON[1], Mika YASUDA[9]

[1] *Center for Integrative Conservation*, *Xishuangbanna Tropical Botanical Garden*, *Chinese Academy of Sciences*, *Menglun*, *Yunnan* 666303, *China*

[2] *College of Forestry*, *Guangxi University*, *Nanning*, *Guangxi* 530005, *China*

[3] *Foundation for Nature Conservation and Preservation*, *Panadura* 12500, *Sri Lanka*

[4] *University of the Chinese Academy of Sciences, Beijing 100049, China*

[5] *Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education*, *College of Life Sciences*, *Zhejiang University*, *Hangzhou*, *Zhejiang* 310027, *China*

[6] *Key Laboratory of Tropical Forest Ecology*, *Xishuangbanna Tropical Botanical Garden*, *Chinese Academy of Sciences*, *Menglun*, *Yunnan* 666303, *China*

[7] *Faculty of Science, Universiti Brunei Darussalam*, *Jln. Tungku Link, Gadong BE*1410, *Brunei Darussalam*

[8] *School of Biological Sciences*, *University of Adelaide*, *Adelaide*, *South Australia* 5000, *Australia*

[9] *Forestry and Forest Products Research Institute*, *Tsukuba*, *Ibaraki* 305-8687, *Japan*

ABSTRACT

Owls have the potential to be keystone species for conservation in fragmented landscapes, as the absence of these predators could profoundly change community structure. Yet few studies have examined how whole communities of owls respond to fragmentation, especially in the tropics. When evaluating the effect of factors related to fragmentation, such as fragment area and distance to the edge, on these birds, it is also important in heterogeneous landscapes to ask how 'location factors' such as the topography, vegetation and soil of the fragment predict their persistence. In Xishuangbanna, southwest China, we established 43 transects (200 m×60 m) within 20 forest fragments to sample nocturnal birds, both visually and aurally. We used a multimodel inference approach to identify the factors that influence owl species richness, and generalized linear mixed models to predict the occurrence probabilities of each species. We found that fragmentation factors dominated location factors, with larger fragments having more species, and four of eight species were significantly more likely to occur in large fragments. Given the potential importance of these birds on regulating small mammal and other animal populations, and thus indirectly affecting seed dispersal, we suggest further protection of large fragments and programs to increase their connectivity to the remaining smaller fragments.

Keywords: Forest fragmentation; Landscape ecology; Nocturnal birds; Owls; Trophic cascades

INTRODUCTION

The majority of the world's species are found in tropical forests, which have been lost rapidly due to anthropogenic activities such as agricultural expansion, logging and urbanization (Haddad et al., 2015). Among tropical regions, Southeast Asia has received particular attention as a priority region for conservation because of high deforestation rates and greater species richness (Sodhi et al., 2010). For example, in parts of Southeast Asia such as Xishuangbanna Prefecture, China, agricultural crops, and specifically rubber plantations, have expanded rapidly during the past thirty years, with a serious loss of forest habitat (Li et al., 2008).

Deforestation produces three interconnected problems for biodiversity: habitat loss, habitat fragmentation and habitat degradation (Fahrig, 2003). A large literature, especially

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*Corresponding authors, E-mail: eben.goodale@outlook.com; quan@xtbg.ac.cn

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extensive for birds, has investigated how 'fragmentation factors' (e.g., fragment size, shape, isolation, and percentage of edge) influence biodiversity, finding mostly negative effects on habitat specialists, insectivores and large frugivores, but positive effects on some generalist species (Bregman et al., 2014; Chang et al., 2013; Matthews et al., 2014). Forest fragmentation is also known to disconnect small populations of organisms from the larger population, leading to a reduction in genetic variation (Hagen et al., 2012). At the same time, in a heterogeneous landscape, 'location factors' (e.g., the topographical position of a fragment and characteristics of its vegetation and soil) could also significantly influence the biodiversity of fragments (Liu & Slik 2014).

Here, as part of an investigation comparing fragmentation and location factors on birds and herpetofauna of Xishuangbanna, we report on the response of a nocturnal bird community to fragmentation. Xishuangbanna is a highly heterogeneous region, with undulating terrains and patchily distributed limestone soils (Tang et al., 2012), and recent studies have suggested that location factors are more important than fragmentation factors in explaining the persistence of trees in fragments (Liu & Slik 2014). We selected nocturnal bird species because of how their presence or absence as predators could affect other species in a fragmented landscape (e.g., Terborgh et al., 2001). Large owls may act as the top predators in some ecosystems, and smaller owls may play a role in the regulation of small rodents, herpetofauna and larger insects (Mikkola, 2014). Although studies on large owls, such as Northern Spotted Owl (*Strix occidentalis*), were important in the development of the fragmentation literature (e.g., Lande, 1987), less is known about how fragmentation affects small owl species, and species-rich communities of owls, especially in Asia. Also, while there has been considerable work on the effect of fragmentation on birds in the region (e.g., Chang et al., 2013), no study has focused on nocturnal birds or raptors. We hypothesized that owls would show stronger responses to fragmentation factors than to location factors, because they maintain large territories. We hypothesized that some owl species (especially those that are large bodied, or habitat specialists) would be influenced negatively by fragmentation factors, particularly decreasing fragment size.

MATERIALS AND METHODS

Study area

The study was conducted within a 10 km radius circle centered on Xishuangbanna Tropical Botanical Garden (XTBG, N21°55′, E101°15′), a research institute of the Chinese Academy of Sciences, located in the Menglun township of Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China (Figure 1). Xishuangbanna is bordered by Laos from the south and Myanmar from the southwest and lies within tropical Southeast Asia (Corlett, 2014), with some characteristics of the subtropics (Zhu et al., 2006). The climate is mainly governed by two seasons: dry, from November to April, and wet, from May to October (Cao et al., 2006). The landscape is a mosaic of mostly rubber plantations, with some banana plantations in river catchment areas, and a few large nature reserves with scattered forest patches, varying in sizes and shapes.

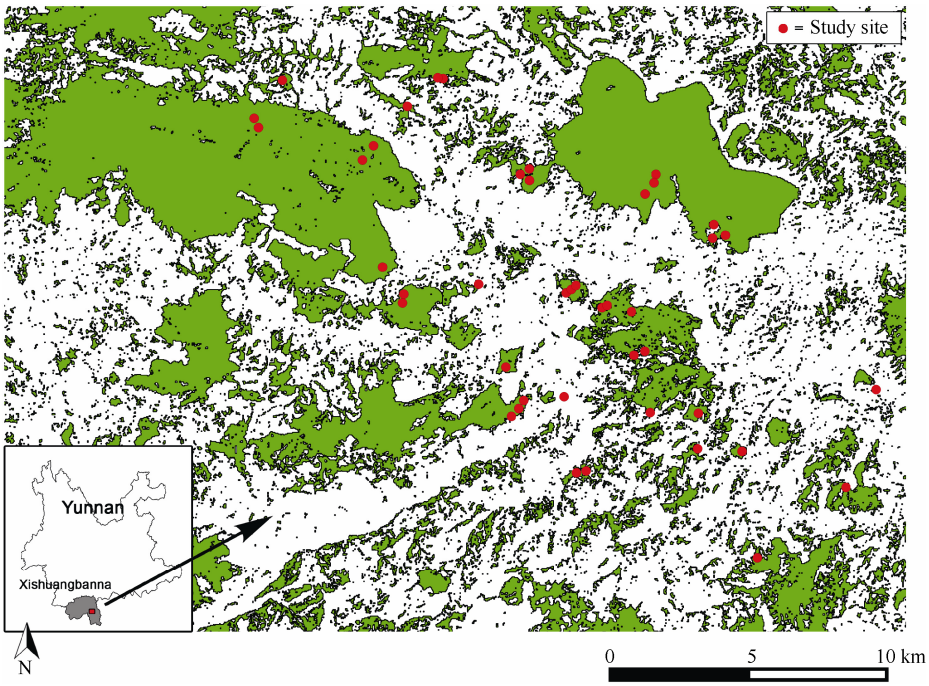

Figure 1 Map showing the study area, natural forest areas (in green) and the 43 sites (red)
The red circle symbol for the sites is not to scale.

Nocturnal bird survey

We selected 43 plots from the 50 vegetation sampling plots, established by the study of Liu & Slik (2014), and established 200 m transects in length inside them. Nocturnal bird surveys were conducted within 30 m on both sides of the transect. We placed the transects on the access paths for the vegetation plots, so that the minimum distance between the starting point of the transect and the forest edge was 25 m for the small fragments (*n*=14 fragments<100 ha) and 100-200 m for the other transects, and the center of the transect was the center of the vegetation plot. Transects were at least 250 m away from each other. These 43 transects were located in a total of 20 fragments that ranged between 0.45 ha and 1 3837.3 ha in size (including two large nature reserves of mean 5 720.3 ha, and 18 unprotected fragments of mean 307.8 ha±483.4 *SD*). The midpoint elevation of transects ranged between 541 and 1 477 m a.s.l.. As transects in the same fragment were not independent of each other, we used a mixed model, with fragment as a random factor (see "Data analysis" below).

We conducted visual and playback encounter surveys (VES & PES), which are the most effective sampling methods for nocturnal birds (Kissling et al., 2010). During the first sampling effort (September-October 2014), two observers (SKD and one local assistant) walked on transect for one hour, searching visually and aurally for nocturnal birds. When we conducted the second sampling (July-August 2015), effort was intensified, applying playbacks of territorial calls of all owl species (8) and nightjars (2) known to occur in the study area, according to MacKinnon and Phillipps (2000). We downloaded recordings from Xeno-Canto online bird calls repository (http: //test. xenocanto.org/). For each species we used three different recordings, selected by their good signal-to-noise ratio, and played them consecutively. We played first calls of small owls and then proceeded to larger owl species, in case the calling of small owls was inhibited in the presence of larger ones, with 2 min intervals between species. All sampling was conducted between 1900h-2400h.

Explanatory and response variables

We collected data on the different transects, with these variables grouped as either 'fragmentation', 'location' or 'degradation' factors. The fragmentation factors were: (1) fragment size in which transects were located, (2) fragment area/perimeter ratio, (3) distance from the edge to the center of transects (shortest projected horizontal distance) and (4) isolation, using the mean proximity index (PROX_MN). PROX_MN measures the degree to which patches are isolated from other patches of the same cover class within a specified search radius (McGarigal et al., 2012; Šímová & Gdulová, 2012; Turner et al., 2001). We used a 2 km search radius because it was large enough for all points to have neighboring patches, and calculated PROX_MN in FRAGSTATS v. 4.0 (McGarigal et al., 2012). The other fragmentation variables were measured using data from Liu & Slik (2014; for further details, please see that article).

The location factors considered were: (1) forest type, where forests were classified into three categories that are nonoverlapping in their tree communities in multivariate analyses (mixed (*n*=15), oak (*n*=16) and limestone (*n*=12, Supplemental Figure 1; note that forest type also is associated with soil type, being either limestone or not), and (2) topology (as three categories; valley (*n*=13), mid-slope (*n*=14) and ridge (*n*=16)). We also considered the degradation factors of: (1) disturbance (transects were considered disturbed if we observed logging and/or ginger planting in them) and (2) whether a transect was in a nature reserve or not. The fragmentation, location and degradation factors collectively were considered as fixed factors in a mixed modeling approach in our data analysis; a summary of the values of these factors for fragments of different sizes is included as Supplemental Table 1. As response variables, we considered species richness and presence-absence of individual species of nocturnal birds.

Table 1 The species detected in the study

Owl species	Body size (cm)	V	A	Slope	*SE*	*P*
Asian Barred Owlet (*Glaucidium cuculoides*)	23.5	30	42	0	N/C^{+}	1.0
Brown Hawk Owl (*Ninox scutulata*)	30.0	4	4	–0.16	0.16	0.31
Brown Wood Owl (*Strix leptogrammica*)	47.5	2	18	0.51	0.23	0.031*
Collared Owlet (*Glaucidium brodiei*)	16.0	3	13	1.06	0.46	0.021*
Collared Scops-owl (*Otus lettia*)	24.5	2	16	0.13	0.12	0.30
Mountain Scops-owl (*Otus spilocephalus*)	19.0	0	22	1.20	0.69	0.08
Oriental Bay Owl (*Phodilus badius*)	26.0	11	5	0.69	0.26	0.008**
Spot-bellied Eagle Owl (*Bubo nipalensis*)	57.5	0	4	1.84	0.81	0.023*

Each species' body size (head to tail; Mikkola, 2014), sample size (V: number of transects on which the species was visually detected; A: number of transects on which the species was aurally detected and/or responded to playbacks), and regression coefficient ('slope') and associated statistics for a generalized linear mixed model explaining their occurrence (presence/absence) at 43 sites in 20 fragments. Fragment was considered a random factor in the mixed model. A positive slope indicates greater occurrence in larger fragments. $^{+}$: Calculation not definite because of zero slope. *SE*: Adjusted standard error; *: $P<0.05$; **: $P<0.01$.

Data analysis

Statistical analyses were conducted in R v.3.1.3 (R Development Core Team, Geneva, Switzerland, 2015). We first checked for spatial autocorrelation in species richness by examining Moran's I values constructed from model residuals, using the 'ape' package (Paradis et al., 2004). We did not find significant spatial autocorrelation in our data (P>0.1). We also estimated species richness per transect, using the 'vegan' package (Oksanen et al., 2015); however, we found estimated species richness (e.g., Chao I estimator) to be highly correlated with the original species richness, and we used the original species richness in subsequent analyses. The species accumulation curve at the landscape scale reached an asymptote, demonstrating that all the species in the study area were sampled (Supplemental Figure 2). We ran random effect mixed models with Poisson error structure, using the 'lme4' package (Bates et al., 2014), to determine the effects of forest fragmentation on nocturnal bird species richness; forest fragment identity was included in the model as a random factor. We employed a multimodel inference approach (Burnham & Anderson, 2002) to determine the most important variables that explain the observed variation in species richness, using the 'MuMIn' package (Barton & Barton 2015). MuMIn grades the models according to their Akaike Information Criterion (AIC) with a correction for small sample sizes (AICc); we obtained the subset of models with ΔAICc of less than 4.

We then ran a multivariate generalized linear model with negative-binomial error structure to determine the effects of fragmentation on nocturnal bird community composition in study area, using the 'mvabund' package (Wang et al., 2012). Note that this program does not allow the use of random factors; however, the other analyses in our study showed the influence of the random variable of fragment to be very small (explaining less than one percent of the variation). We assessed the significance of explanatory variables using 999 permutations of a Monte-Carlo test. We used non-metric multidimensional scaling to visualize differences in bird composition, using the vegan package. We also determined the effects of fragment size on the occurrence probability (presence-absence) of each species using generalized linear mixed models with binomial errors and forest fragment as a random factor.

As it is possible that visual detections could be influenced by canopy density, which itself could vary due to the fragmentation, location or degradation factors, we re-analyzed the data using only aural detections, and found qualitatively similar results (Supplemental Table 2).

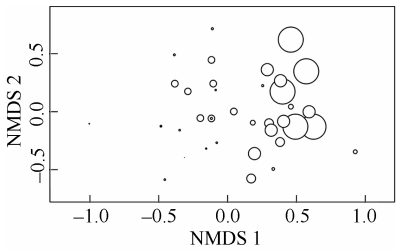

Figure 2 A non-metric multi-dimensional scaling of nocturnal bird assemblages

Fragment size, represented by the diameter of the circles, is related to community composition.

Table 2 Results of the model averaging approach on how owl species richness was influenced by 'fragmentation' and 'degradation' factors ('location' factors were eliminated in the model averaging process)

	Factor	Estimate	*SE*	CI 2.5%	CI 97.5%	Imp	*n*
Fragmentation factors	Fragment size	0.168	0.049	0.070	0.266	1.00	9
	Distance to edge	0.055	0.079	–0.104	0.215	0.22	3
	Isolation (PROX_MN)	0.028	0.110	–0.195	0.249	0.14	2
Degradation factors	Nature reserve inclusion	0.229	0.232	–0.239	0.698	0.31	4
	Disturbance	–0.122	0.184	–0.495	0.249	0.22	3

Estimate: model-averaged coefficients; CI: confidence intervals (2.5% and 97.5%, respectively); *SE*: adjusted standard error; Imp: relative importance of the factor, *n*: number of models with ΔAICc<4 that included the factor; there were 9 such models in total. The adjusted R^2 value for the full model (all factors fragmentation, location and degradation factors included) was 0.40.

RESULTS

We recorded 8 species of owls with 211 individual observations, which include both visual and aural encounters within 30 m of the transects (see Table 1 for species). No nightjar species was recorded during the systematic data collection, but Large-tailed Nightjar (*Caprimulgus macrurus*) was observed in two large fragments actively foraging on insects. Four species of owls, Collared Owlet (*Glaucidium brodiei*), Mountain Scops-owl (*Otus spilocephalus*), Oriental Bay-owl (*Phodilus badius*) and Spot-bellied Eagleowl (*Bubo nipalensis*) were never recorded within forest fragments smaller than 100 ha.

The model-averaged estimates from mixed models indicate that only fragmentation and degradation factors influenced owl species richness; location factors did not appear in any of the models with ΔAICc of less than 4 (Table 2). The factor in the most models was fragment size (9 of 9 models), with transects in larger fragments having more species; this was the only factor for which the 95% confidence interval did not include zero (see Table 2). Reserve status (protected reserves had more

species), disturbance (less disturbed sites had more species), distance to the edge (greater distances had more species), and isolation (less isolated transects had more species) were included in four, three, three and two models, respectively. The multivariate generalized linear model demonstrated that fragmentation was a significant influence on composition (P=0.02), with transects in fragments of the same size clustering together in multivariate space (Figure 2).

The occurrence probability of four owl species increased at transects in larger fragments, with the other four species not having any significant effect (see Table 1, Figure 3). The largest owl recorded was Spot-bellied Eagle-owl (*B. nipalensis*), which was the most sensitive owl species to fragment size, only found in fragments above 3 200 ha.

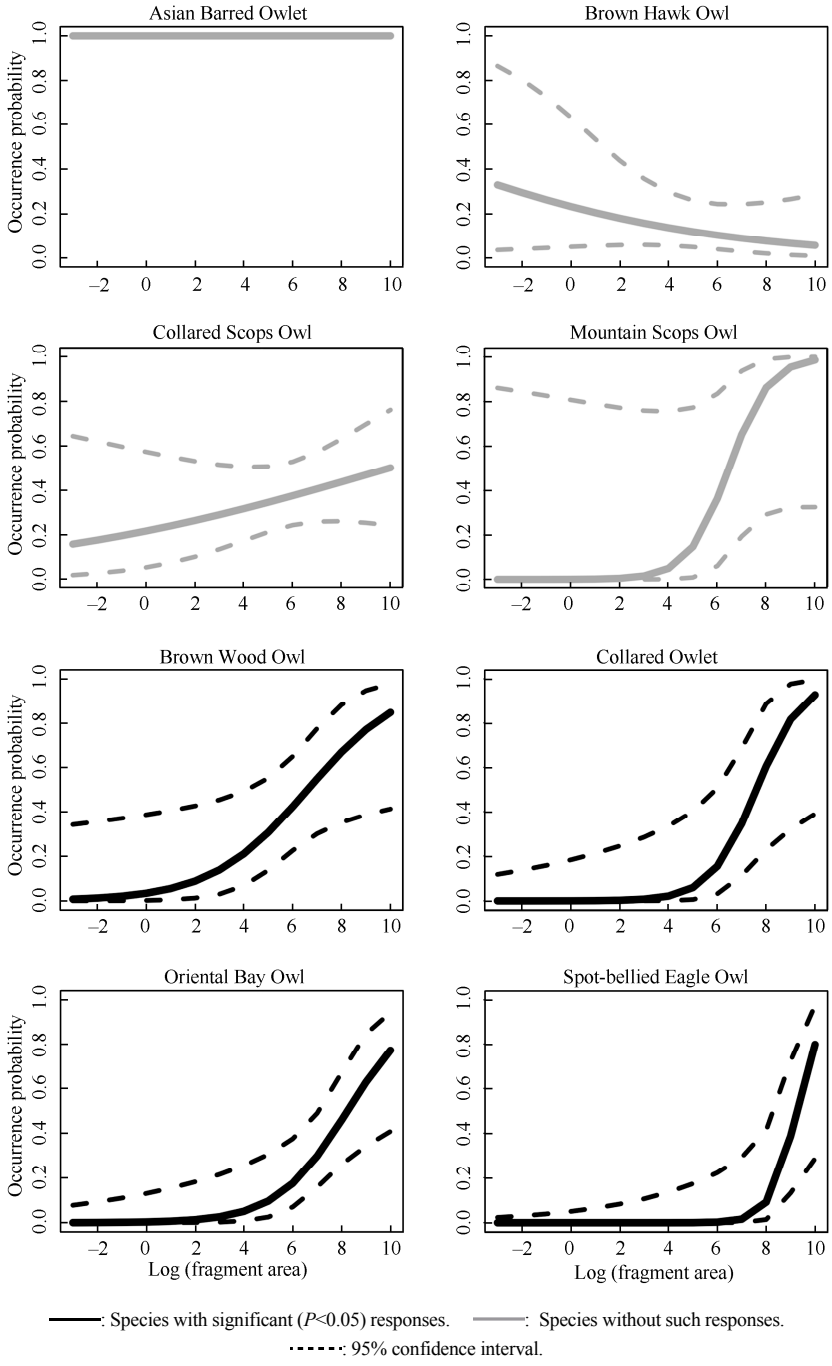

Figure 3 Occurrence probability of each owl species with increasing forest fragment size (log transformed)

DISCUSSION

As expected, our results showed that the fragmentation factors had a dominant effect on owl species richness in this area. The occurrence probability analysis showed that for eight species of owls, four species had a significant positive relationship with increasing fragment size. Small fragments thus appear inhospitable to most of the owl species recorded in this landscape. Several characteristics of small fragments could be driving this effect. Small fragments may not be big enough to support larger territories and prey volumes that the large species require (Mikkola, 2014), and/or small fragments may lack large old trees with hollows to serve as nests (Kavanagh & Bamkin, 1995). Dense canopies (which were poorly developed in the small forest fragments) are also a crucial microhabitat requirement for owls to avoid sunlight/heat, during their retreat/day roost (Hayward & Garton, 1984). It should be noted that both our visits to forest fragments were in the wet season, and owl habitat selection might differ in the dry season. A further limitation of the study is that we do not have data on characteristics of the vegetation, such as the presence of nesting trees or canopy density, that could be used to see exactly how microhabitat differences between transects influence the results.

Some owl species were widely distributed within the study area, with high tolerance to habitat disturbance. For example, Asian Barred Owlet (*G. cuculoides*), was abundant at all sites, and Brown Hawk Owl (*N. scutulata*), known to prefer open landscapes (Olsen et al., 2016), non-significantly declined with increasing fragment size. Kavanagh & Bamkin (1995) and Weaving et al. (2011) also showed that some owl species are tolerant to logging and other anthropogenic disturbances in semi-urban environments. We can conclude that in a nocturnal avian community within any given area, there are some generalist species that have the flexibility to adapt to a changing environment, as well as specialized species that have a higher risk of vanishing locally (Loyn et al., 2001). In this study, we found that fragment size affected not only large-bodied owl species but also smaller owls, including the smallest species in the community, Collared Owlet (*G. brodiei*). This species has relatively large eyes relative to its body length, and may avoid forest edges and the more well-lit parts of the forest, as shown recently in the study of Martínez-Ortega et al., (2014) for large-eyed owl species.

Nocturnal bird communities play an important role in regulating the populations of rodents, herpetofauna and large insects in forests. Most of the recorded small-bodied owl species in this study preyed on large insects; they are opportunistic hunters by nature (Mikkola, 2014). All the recorded large owl species (Spot-bellied Eagle Owl, Brown Wood Owl and Oriental Bay Owl) feed mostly on rodents, small reptiles and amphibians (Mikkola, 2014). Thus the local extinction of these nocturnal avian predators may cause a cascading effect on the food webs of the small fragments. In particular, the lack of these large owl species may trigger an increase in rodent populations. Indeed, some studies have shown high densities of rats in small fragments (Gibson et al., 2013). A high density of rats, in turn, may directly influence seed germination, as rodents are known to be seed predators as well as dispersers (Heithaus, 1981; Loayza et al., 2014).

Given the positive effect of fragment size on owl community in our study, we strongly recommend that conservation efforts preserve large fragments in this area and work towards connecting smaller fragments with larger ones. Large-bodied owls have been used with success as 'umbrella species' for conservation in northern temperate countries (Lamberson et al., 1994; Loyn et al., 2001), and perhaps more diverse tropical assemblages of owls can be used both as bio-indicators of environmental health, and as educational tools for increasing support for conservation.

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SUPPLEMENTARY MATERIALS

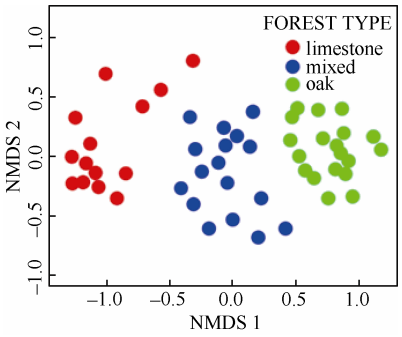
Supplementary Table 1 Characteristics of the transects as to the fragmentation, location and degradation factors considered in the analysis

	Transects in small forest fragments (*n*=14)	Transects in other forest fragments (*n*=18)	Transects in nature reserves (*n*=11)
Fragmentation factors			
Mean fragment area (ha)	31.30	753.64	5720.25
Mean area/ perimeter ratio	0.0363	0.0140	0.0123
Mean distance to the edge (m)	70.3	147.5	315.3
Mean isolation index (PROX_MN 2km)	62.64	242.07	267.52
Location factors			
Forest types (M: mixed, O: Oak, L: Limestone)	M=43%, O=43%, L=14%	M=11%, O=39%, L=50%	M=73%, O=14%, L=09%
Topography (V: valley, MS: Mid-slope, R: ridge-top)	V=21%, MS=43%, R=36%	V=22%, MS=28%, R=50%	V=55%, MS=27%, R=18%
Degradation factors			
Disturbed proportion	57%	50%	36%

Supplemental Table 2 Model averaging results (when only data from aural detections were used)

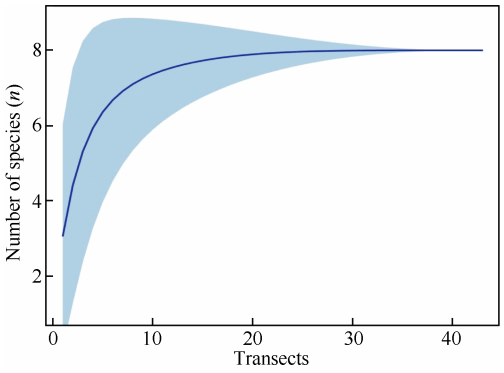
	Factor	Estimate	CI 2.5%	CI 97.5%	*SE*	Imp	*n*
Fragmentation factors	Fragment size	0.167	0.072	0.261	0.048	1.00	5
	Distance to edge	0.024	–0.137	0.186	0.083	0.13	1
	Isolation (PROX_MN)	0.021	–0.204	0.246	0.115	0.12	1
Degradation factors	Nature reserve inclusion	0.211	–0.260	0.683	0.240	0.18	1
	Disturbance	–0.129	–0.501	0.242	0.189	0.16	1

This Table is similar to Table 2 in the text, except it uses a subset of the data. See Table 2 for explanation of abbreviations. The adjusted R^2 value for the full model was 0.39.



Supplemental Figure 1 Forest type of the study site, with three relatively discrete categories: limestone forests, mixed forests and oak forests

Here tree species composition data is used to show that these forest types do not overlap each other. The NMDS shows the tree diversity at the vegetation plots that were in the same locations as our transects.



Supplemental Figure 2 Species accumulation curve for nocturnal birds, detected at 43 transects located in 20 fragments

The shaded area depicts 95% confidence intervals. The lack of increase of the accumulation curve after 20 transects suggests that all the species in the area during the study period were detected.

Conservation education and habitat restoration for the endangered Sagalla caecilian (*Boulengerula niedeni*) in Sagalla Hill, Kenya

Patrick K. MALONZA*

Herpetology Section, *National Museums of Kenya*, 40658-00100 *Nairobi*, *KENYA*

ABSTRACT

The Sagalla caecilian (*Boulengerula niedeni*) is an endangered amphibian endemic to Sagalla Hill in the Taita Hills. This burrowing worm-like species prefers soft soil with high moisture and organic matter. The major threats to the Sagalla caecilian are soil erosion caused by steep slopes, bare ground and water siphoning/soil hardening from exotic eucalyptus trees. The purpose of this study was to get a better understanding of the local people's attitude towards this species and how they can contribute to its continued conservation through restoration of its remaining habitat. In this study, it was found that 96% of Sagalla people are aware of the species, its habits and its association with soils high in organic matter. It was also found that 96% of Sagalla people use organic manure from cow dung in their farms. Habitat restoration through planting of indigenous plants was found to be ongoing, especially on compounds of public institutions as well as on private lands. Although drought was found to be a challenge for seedlings development especially on the low elevation sites, destruction by livestock especially during the dry season is also a major threat. In this study, it was recommended that any future habitat restoration initiative should include strong chain-link fencing to protect the seedlings from livestock activity. Recognizing that the preferred habitats for the species are in the valleys, systematic planting of keystone plant species such as fig trees (*Ficus*) creates the best microhabitats. These are better than general woodlots of indigenous trees.

Keywords: Local people; Caecilian; Awareness, Protection, Habitat rehabilitation

INTRODUCTION

The Sagalla Caecilian (*Boulengerula niedeni* Müller, Measey, Loader & Malonza, 2005) is an elongated, slender, brownish, limbless, tailless, soil-burrowing amphibian about 300 mm in body length (Figure 1). Its colour varies depending on the microhabitat substrate, with specimens in well-shaded sites e.g., between banana plants and in high altitude areas tending to be dark brown, whereas those in lower elevations being light brownish. Superficially it resembles a large earthworm, but differs by its serpentine mode of movement while on the soil surface. Due to its snake-like movement many people on first sight think that it is a snake. It differs from snakes by having moist smooth skin, segmented body and a pointed pigment less head as opposed to snakes, which have dry, scaly skin, with pigmented heads.

The Sagalla caecilian is endemic to Sagalla Hill, one of the mountain blocks of the Taita Hills (Müller et al., 2005). On Sagalla Hill, *Boulengerula niedeni* is found in low densities in farms and patches of indigenous forests (<3 ha remaining) at altitudes of between 1 000-1 500 m (Malonza, 2008; Malonza et al., 2010; Malonza & Veith, 2012). The caecilian prefers moist, organically rich soft soils. In farmlands, these caecilians can be found mainly in soils rich in organic manure or under organic debris, the base of terraces, under fig trees and along the edge of streams; whereas, in the indigenous forest it is common in decomposing dead logs, at the base of palm plants and in forest leaf litter (Malonza, 2008; Malonza et al., 2010). Since 2014 it is globally categorized in the IUCN Red list as Endangered but before then as critically endangered. In the entire Sagalla Hill highland region the proportion of its preferred habitat is very small. There is continuing decline in its area of occupancy, extent and quality of habitat due to increased

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*Corresponding author, E-mail: kmalonza@museums.or.ke

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human population. The current estimated extent of occurrence is less than 20 km^2 and area of occupancy of the fragmented sites is about 7.06 km^2 (Malonza et al., 2010).

Figure 1 Sagalla caecilian *Boulengerula niedeni* (in life) (upper) and its general habitat (lower)

By preferring organically fertile moist soft soils for burrowing, its major threat is soil erosion and compaction or hardening. Hence all activities that promote soil erosion (e.g., poor farming practices), hardening and drying of soil (e.g., from eucalyptus trees), results in the decline of the caecilian population. Currently the high slopes of Sagalla Hill are covered with exotic eucalyptus and pine trees (Sagalla forest plantation ca. 70 ha) planted around 1956. During its growth over the years eucalyptus trees in particular (due to their water draining habits) have resulted in drying out of springs and hardening of the soil surface reducing the amount of habitat suitable for the Sagalla caecilian. These negative effects of eucalyptus on ground water has also been witnessed elsewhere in India (e.g., Joshi & Palanisami, 2011)

In conservation of this species the main challenge is how to convince the local people to conserve the caecilian habitat, as they view the caecilian as having no direct tangible benefit. In order to conserve the species and restore its habitat, conservation education and awareness is important. Given that the species superficially resembles both an earthworm and a snake, there is need to get a clear understanding of the attitude and perception of the local people toward the caecilian so as to promote conservation of the species and its habitat. The fact that the Sagalla caecilian prefers organically moist fertile soil means that the species is an indicator of good healthy soil required by the local farmers for crop production. The species is actually an ecosystem service provider in that it helps with organic matter decomposition and soil aeration, both important for seed germination and seedling development. This means both the local farmers and the caecilian require organically rich soil for their survival, resulting in a form of mutualism. One way to achieve this would be through a conservation campaign for the local people to understand the species biology, environmental importance, threats and conservation initiatives. This campaign should inform the locals about the tangible threats to their survival, e.g., soil erosion and water siphoning effects of Eucalyptus, which can be justified by a cost-benefit analysis over time. Consequently in the process of mitigating the causes of these threats for their own benefits, they will be indirectly conserving the Sagalla caecilian by restoring or improving its habitat. One of the ways to improve the species habitat is through replanting indigenous trees on Sagalla Hill. One of the main objectives of this study was to create awareness by demonstrating the link between human requirements and those of the Sagalla caecilian, thereby, changing peoples' perception of the species towards a more positive orientation that will, in turn, aid in the conservation of the environment for both human and the amphibian.

MATERIALS AND METHODS

Study area

Education and awareness surveys were done during three visits; 22 March-05 April 2011; 04-18 December 2011 and 05-21 March 2012 in the highland area of Sagalla hill. Sagalla hill is one of the mountain blocks of the Taita Hills, which is the northern-most block of the Eastern Arc Mountains (Lovett, 1990). The Eastern Arc Mountains is one of the sections of the Eastern Afromontane biodiversity hotspot in Africa (Sloan et al., 2014). Sagalla hill is a dry land rocky hill located close to Voi town (S3°23′, E38°34′, 560 m a.s.l.) separated from the town by the Mombasa highway (Figure 2). It rises from an altitude of about 600 m a.s.l. from the Tsavo plains to about 1 500 m a.s.l.. The lower slopes of the hill are covered by *Acacia-Commiphora* bushland and woodland giving way to a sub-montane vegetation zone at about 1 000 m a.s.l. and above. The highland area of the hill is densely populated by small-scale farmers mainly growing crops and fruit plants such as; maize, bean, banana, sweet potatoes, pawpaws, citrus, avocado and mango. The upper slope of the hill (mainly above 1 300 m a.s.l.) is covered with exotic eucalyptus and pine trees.

Survey sites

The following major sites and their environs were visited: (1) Mwalangi market area (S3° 30.657′, E 38°34.593′, 1 095 m a.s.l.), Sagalla

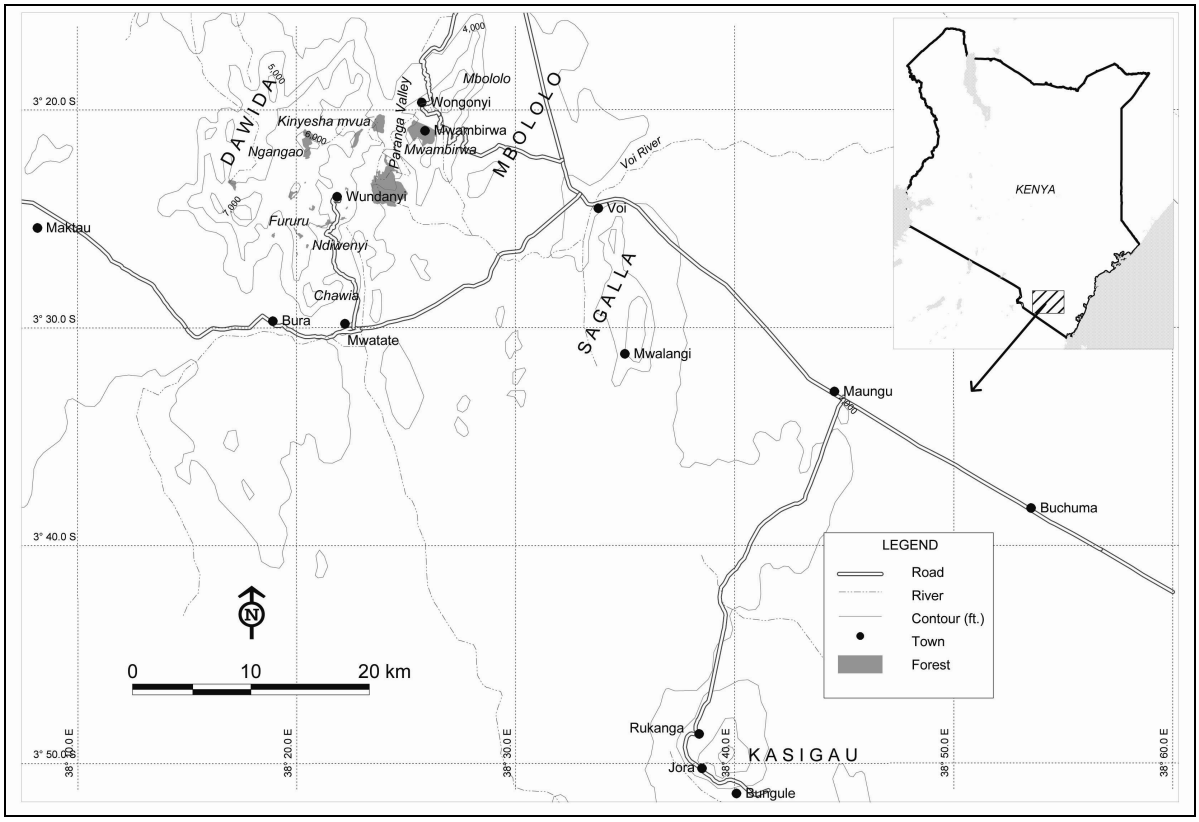

Figure 2 Map of the Taita Hills showing the main block Dawida and the three isolates of Mbololo, Sagalla and Kasigau (Inset a map of Kenya showing the location of Taita Hills) (adopted from Malonza et al., 2010)

chief camp (S3°30.769′, E38°34.533′, 1 102 m a.s.l.), Wray primary school and Mwakichuchu mixed secondary school (S3°30.587′, E38° 34.574′, 1 108 m a.s.l.); (2) Talio primary school (S3°31.641′, E38° 34.841′, 1 080 m a.s.l.); (3) Mlondo primary school, Sagalla health centre, Our Lady of Perpetua Succour Girls Secondary School (S3° 29.810′, E38° 34.913′, 1 108 m a.s.l.); (4) Kanyanga market area, Sagalla youth polytechnic (S03° 28.937′, E38°34.974′, 1 098 m a.s.l.); (5) Kizumanzi primary school, Serengi valley (S3°28.579′, E38°34.213′, 1 137 m a.s.l.); (6) Mghange area (S3°29.403′, E38°35.649′, 1 260 m a.s.l.): (7) Sagalla primary school, Kishamba area (S3°29.770′, E38°35.698′, 1 387 m a.s.l.); (8) Marie primary school (S3°31.064′, E38°35.553′, 1 418 m a.s.l.), Dambi area (S3°31.063′, E38°34.968′, 1 249 m a.s.l.); (9) Kwen-Tole area near the forest Sagalla forest station (S3°30.514′, E38°34.952′, 1 300 m a.s.l.); (10) Suluni-Bule area, upper Kizumanzi catchment (S3°29.126′, E38°35.585′, 1 332 m a.s.l.).

Research methodology

In this social survey a structured questionnaire was used to collect data on various aspects by employing the best practice as recommended by White et al. (2005). Respondents or interviewees were randomly selected within the general area of Sagalla where the Sagalla Caecilian is known to inhabit.

Community conservation education and awareness

This survey was done during the wet seasons of March-April and November-December, a period when caecilians are easily found due to their dependence on soil moisture. In the first survey (22 March-05 April 2011), a structured questionnaire was administered randomly by visiting and interviewing local people in their farmlands or homesteads. The respondents were asked questions ranging from whether one knows the species; its new Ki-sagalla name of *"Kilima-mrota"* and its habits (see Appendix 1A). Only one person was interviewed at a time and when more than one where found together only one was interviewed and educated as the rest listened. However, at the end all present were allowed to ask questions for general education and awareness creation. Most of the people interviewed were those found farming except on Sundays when majority were at home (before or after church service) and not in farms. In addition, group education and awareness of the species biology, importance, threats and conservation were given to schools and public meetings. For general awareness all the six primary schools (Wray, Talio, Mlondo, Kizumanzi, Marie and Sagalla), and Mwackichuchu mixed day and boarding Secondary School were visited. One education/ awareness session was given at a public meeting of about 200 people organized by the Sagalla location administrative chief at Kanyanga market.

In the second awareness survey (04-18 December 2011) the respondents were randomly chosen as they were encountered and asked additional more specific questions to those of the first survey, such as; species micro-habitats, importance, threats and habitat conservation methods and the execution procedure followed was similar to that of the first survey (Appendix 1B). However, most of the people interviewed were those within their homesteads because during this period many had finished weeding and were not available within the farmlands. During both the first and second interviews a live caecilian was carried for hands-on demonstrations. In general, surveys were conducted systematically but respondents were chosen randomly in most of the Sagalla areas where the species is known to occur.

In the third and last survey, the major aim was to take stock of major activities done so far by the various institutions, government agencies, and among other organizations within Sagalla that promote the conservation of the caecilian and its habitat. These include; planting indigenous trees, replacing exotic trees, e.g., eucalyptus, soil erosion prevention, e.g., terracing, increased use of organic manure, and public education on the species among others. The head or a representative of all the six primary schools, three secondary schools (Mwakichuchu, Sagalla Girls' and Kizumanzi), Sagalla Youth Polytechnic, Sagalla Health Centre, Wray Memorial Museum, Sagalla Forest Station, and Taita-Taveta Wildlife Forum were asked to point-out what their institutions have done so far from a given checklist (Appendix 1C). Two sets of laminated posters (A0 mostly English) and A4 (with Kiswahili translation) of summarized information on the species where distributed to all the institutions. Only data from the structured interviews was used in the analysis while information from public group education was used in a descriptive way where applicable.

Habitat restoration

This was done during the rainy seasons of November-December and March-April mainly between November 2012 and April 2015. The purpose was to restore and enhance the quality of habitat for the Sagalla caecilian and ultimately increase the species population size. The goal is to reduce the direct threats to the species, namely soil erosion and water draining or siphoning effects of exotic plants especially eucalyptus trees in the area. Indigenous tree seedlings were sourced from local people's tree nurseries. They were then planted in collaboration with local people under the supervision of a Sagalla resident and Taita-Taveta Wildlife Forum field officer. Planting was done on public institutions compounds including all the primary schools, Youth polytechnic, St. Marks ACK Church/Wray Memorial Museum, Sagalla chiefs' camp, Lata Dam and on farmlands of members of interested farmers.

Data analyses

Data was analysed with chi-square-Test to test whether the respondents' knowledge or awareness of the caecilian biology and its conservation depends on their level of education or age. The respondents' level of education was determined by asking their level of education (primary, secondary, colleague/university or none for those who have not entered as formal education class. Age was determined from respondents' who provided their respective age in years.

RESULTS

The respondents

In the first survey a total of 89 (43 males, 46 females) respondents were interviewed while in the second survey the total was 90 (42 males, 48 females). On education 20 years and below was the baseline for primary and secondary while between 20 and 60 plus for those above secondary in tertiary level (college and University). Considering these in the analysis the average age of 40 years (above or below) and secondary education level were used as reference points to determine the interviewee response. In Kenya the age of 20 years is the average youthful age while 60 years is the retirement age from active work. So the average i.e., 40 is considered the prime or most productive age class.

How many know the Sagalla caecilian?

The Sagalla people very well know the Sagalla caecilian as observed from the first (96%) and second (96.5%) surveys, respectively. Even those who claimed not to know when shown a live specimen acknowledged that they had seen it. However, proportionally few people (26%) have heard or are aware of the new Ki-Sagalla name for the caecilian, i.e., *Kilima-mrota*. Those who were aware heard it mainly from their neighbours (40%). Others heard from the 2006 Wray primary school name search competition pupils, Taita-Taveta Wildlife Forum Officer, grandmother, visitors searching for the caecilian, and visiting Tsavo National Park staff or seen from my first survey mini-poster (flier) posted on the Chief's camp notice board. Only 11% had heard or learnt about the name from school pupils or students. Therefore, many on the first sight still call it *Mng'ori* or *mwamng'ori* a general Taita name that refers to a large earthworm. However, a good number (41%) can differentiate it from an earthworm and even blind snakes.

The attitude and beliefs of people towards the Sagalla caecilian

Despite the caecilian's superficial resemblance to a snake majority (85%) of the respondents said they did not kill them on sight as they are harmless and some (39%) could point out the species importance in helping organic decomposition and only killed accidently if cut while tilling or weeding. Only 9% said that they purposely killed them because they look like snakes and think that they can bite or eat/cut roots of sweet potatoes and other food crops. However, 63% of those who normally kill the caecilians said they will stop after understanding that they are harmless and constitute a very important part of the environment by helping in soil aeration, organic manure decomposition and feeding on termites that are crop pests.

Where and when do you find caecilians?

A proportionally large number of Sagalla people (90% and 94% of the first and second survey respondents respectively) clearly

know where and when to find Sagalla caecilians. Most of them (95%) clearly associated the species with wet areas and easily found mainly during the rainy season within microhabitats organically fertile soils. In Sagalla farms such microhabitats include base of bananas, terraces, and base of remnant *Ficus* trees and palm plants, near cattle sheds, along water streams including those fringed with indigenous plants or the exotic and invasive shrubs like *Tithonia diversifolia* and *Lantana camara*. When asked whether they use organic manure or artificial fertilizers, almost all (96%) said that they prefer and will continue using the organic (mainly cow dung manure). Many clearly pointed out that they have never used artificial fertilizers.

The influence of age and level of education on knowledge of the species habitat requirements, importance, threats and conservation initiatives

Results from December 2011 data, showed that the respondents who were below or above 40 years of age, were 44% and 38% respectively knowledgeable but with no statistically significant difference (χ^2 test, df_1=1.17, P=0.28). In reference to the level of education it was found that the 55% and 29% of the respondents whose education level was below and above secondary school respectively were knowledgeable but with no significant difference (χ^2 test, df_1=1.98, P=0.16).

Knowledge on importance of the Sagalla caecilian to the environment

Information was sought from the respondents on their knowledge on the importance of the Sagalla caecilian. Some of the expected responses included helping in soil aeration and decomposition of organic manure, pest control (eat termites), and a symbol of high organic soil fertility. The majority (61%) seemed unaware of any importance of this species to the environment. However, some (39%) seemed to point out a few of them which they learned from neighbours and/or environmental education seminars. Again after understanding the species microhabitat requirements, the majority (86%) could point out some of the activities for protecting the species and its habitat. These include; terracing, not burning farm trash, not killing, increased use of organic manure, planting indigenous trees and banana plants.

Knowledge on threats to the Sagalla caecilian

Almost all (99%) the Sagalla people interviewed were aware of the water sucking (a negative impact) effects of the eucalyptus trees. In addition, after getting a clear picture of the habitat requirements of the species, over half (57.5%) of the respondents could point out some of the things threatening the survival of the caecilians.

Majority of the people (95%) preferred planting indigenous over exotic trees. Some of their reasons for this choice are that indigenous trees increase soil moisture, add humus, and have medicinal value. Other trees, like *Ficus* trees, do not inhibit crops growing underneath. The few who preferred exotics pointed out *Grevillea robusta* trees that do not have inhibitory effects on crops and adds to soil fertility. Some also mentioned planting of exotic fruit plants and planting of exotics for timber only within wastelands.

Habitat restoration for conservation of the Sagalla caecilian

During the last assessment on current conservation activities for the species 93% of the institutions had at least planted indigenous trees within their compounds. The native tree, *Croton megalocarpus*, was found to be the most successful due to its fast growth and resistance to drought. This tree and other native trees could also be seen within people's homesteads. Two primary schools (Sagalla and Marie) in the high altitude areas, had replaced almost their entire exotic species with indigenous trees. However, the challenge was the intensive sprouting of young eucalyptus saplings in Marie primary school after deliberate burning. In general, a good number of institutions (57%) had even replaced some of their exotic pine and eucalyptus trees with native species. Most of the institutions and/or their representative interviewed had done at least some of the activities that promote the conservation of the Sagalla caecilian and/or their habitat (Table 1). During the current habitat restoration programme over 10 000 seedlings have been planted in most of the schools and other institutions.

Table 1 Results of the conservation initiatives assessment of 14 institutions during the March 2012 survey

Initiative	Percentage (%)
Planting indigenous trees on communal/private lands	93
Replacing exotic plants (e.g., Eucalyptus, pine) with indigenous trees	57
Farm terracing and reinforcing with Napier grass or its equivalent, e.g., stone piles among others ways	86
Planting more banana plants as a source of food and control of surface runoff	43
Increased use of organic manure (cow-dug, compost, farm trash)	78
Not burning farm trash but letting it to decompose	93
Educating others on the presence and importance of the Sagalla caecilian in the environment	71

DISCUSSION

Over 95% of the preferred habitat for the Sagalla Caecilian is on privately owned land and mostly valley floor farmlands. On Sagalla Hill caecilians have only been found in suitable sites within indigenous forest patches and farms, while none have been found in the exotic plantations of pine and eucalyptus

(Malonza, 2008; Malonza et al., 2010). The exotic plantation was established in 1956 and it is assumed that the caecilians (then not discovered) were there but disappeared due to habitat alteration. From past and current works it is clear that the Sagalla people are, and continue to be aware, of the Sagalla caecilian and the conservation initiatives. Respondents' age and level of education on knowledge of the species habitat requirements, importance, threats and conservation initiatives was found to be insignificant. Therefore, old people and those who have low education level (primary and below) have a lot of knowledge. This implies that knowledge about the species biology, importance, threats and conservation is independent of age and education level. This means that understanding this species only requires indigenous knowledge that can be passed from one generation to another by older experienced generation and not necessarily in school. Basically this means this knowledge is just not taught in schools and the only way people learn about the caecilian is through practical experience by specific educational endeavours since it is not included in the standard curriculum.

This survey shows that very few people purposely kill caecilians because of their resemblance to snakes, but no direct field observation was made of a farmer trying to kill a caecilian. The perception that anything resembling a snake in morphology and movement deserves to die is quite widespread across Kenya (Wojnowski & Malonza, 2010). The local people's preference and continued use of organic manure in farmlands is very encouraging and boosts all efforts for the conservation and protection of the Sagalla caecilian.

Planting and replacing exotic trees both on peoples land as well as on the government forest plantations is the ultimate solution to enhance this species survival., Eucalyptus trees in particular have been found to have negative impacts on the water table by depleting ground water causing the soil to dry up and become hard e.g. in India (Joshi & Palanisami, 2011) and in Ethiopia; (Tilashwork, 2009). The human population in Sagalla is relatively dense and continues to increase. The current work is focussed on replanting indigenous trees in areas owned by public institutions. Most of these institutions are located on high grounds susceptible to soil erosion and unsuitable for caecilians. This decreases the impact of this endeavour, and with the increased human activity within these institutions foot traffic contributes to soil compaction.

The most preferred microhabitats with high abundance of Sagalla caecilian are those on stream valleys (Malonza, 2008). Therefore habitat restoration through planting of indigenous trees in the general area increases the cumulative amount of microhabitats for the caecilian. On private farms the most preferred natural microhabitats are bases of remnant *Ficus* species (fig trees). This means increasing their density will immensely contribute in increasing the species preferred microhabitats. Some Sycamore fig trees, *Ficus sycamorus*, occurred on valleys on the low elevations in Sagalla. This and other fig tree species are known keystone fruit trees attracting a high number of birds and other animals (e.g., frugivores/ insectivores). Droppings of birds and their fruits drop on the base increasing organic manure after decomposition. Fig trees are fast growing, drought resistant, retain soil moisture, live long and increases soil fertility. In addition they do not inhibit crops growing underneath, thus allowing farmers to grow vegetables and other crops. Naturally one mature fig tree can occupy a big area creating a substantial microhabitat that can harbour a viable caecilian population. Traditionally the Sagalla people respect and protect fig trees and these trees also do not produce good fuel wood/charcoal hence left for beauty and/or to increase soil fertility. Therefore, one way for long-term conservation of the Sagalla caecilian lies in increasing the number of fig trees within the stream valleys. This should involve careful and systematic species selection depending on the elevation of a particular site. A small fence can be made around the young seedling to prevent destruction by livestock. Given that they are less preferred by livestock due to their milky sap and are fast growing, they will definitely get established within few years. Because farmers are aware of the importance of this species each can be encouraged to plant at least one seedling depending on the size of their farm.

The long-term success of the conservation of the Sagalla caecilian is through promoting activities that have tangible benefits to the local people and indirectly benefit the caecilians as well. Currently, many Sagalla people are aware of the presence of the Sagalla caecilian in their area. However, you can still find a number of people with little information about the species habits, threats and conservation of the caecilian and its preferred habitat. To make education and awareness campaign an ongoing process AO, A2, A3 and A4 laminated posters were distributed in all institutions including the village headmen through the area administrative chief.

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APPENDIX 1 The questionnaires and the checklist used during the farmers and institution interviews

A Questionnaire I

Site details: …… **Date:**……

1. Respondent gender: i) Male ii) Female
2. Your age group in years:
   i) Less than 20 ii) 21-30 iii) 31-40 iv) 41- 60 v) over 60
3. Your education level:
   i) Primary ii) Secondary iii) College/University iv) None
4. Do you know the Sagalla caecilian? Yes/No
5. If Yes! Are you aware of its new Ki-sagalla name (*Kilima-mrota* = burrowing and slender)? Yes/No
6. If No! Do you think the name denotes/or is suitable for the caecilian? Yes/No
7. If No! Are you willing to start using its new name over the general local name of *Mng'ori* which refers to both caecilians and earthworms for its identity? Yes/No
8. Do you normally kill these caecilians on sight? Yes/ No
9. If Yes/No! Why (Please specify) ……..……
10. Would you stop killing these caecilians if informed that they are harmless? Yes/ No
11. In which micro-habitats do you find caecilians? i) Everywhere under soil ii) others (Please specify) ……..
12. Are you aware that they mostly prefer soils high in organic manure? Yes/ No
13. If Yes! Are you aware that this shows that they are good indicators of high soil fertility? Yes/No
14. Are you aware that organic manure (animal manure) is cheaper and lasts longer in soil than inorganic manure (fertilizers)? Yes/No
15. Are you aware about the water draining effects of eucalyptus trees? Yes/No
16. If you are informed that this caecilian occurs **only** in Sagalla and nowhere else in the world, would you be proud to protect it and its habitats for posterity? Yes/No
17. Would you prefer planting indigenous or exotic trees in your farm? Indigenous, exotic. Others (specify) ……..
18. With this knowledge about the uniqueness and importance the Sagalla caecilian would you advocate to all Sagalla people for its continued protection? Yes/No

B Questionnaire II

Site details: …… **Date:** ……

1. Respondent gender: i) Male ii) Female
2. Your age group in years: i) less than 20 ii) 21-30 iii) 31-40 iv) 41-60 v) 51-60 vi) over 60
3. Your education level:
   i) Primary ii) Secondary iii) College iv) University v) None
4. Do you know the Sagalla caecilian? Yes/No
5. If yes: How can you differentiate it from an earthworm/blind or worm snake? Are you aware of its new Ki-sagalla name "***Kilima-mrota***"? Yes/No
6. If yes! How did you come to know the name? i) School pupils/student ii) Another farmer/neighbour iii) Baraza, iv) others (Please specify) Where (sites) and when (season) do you normally find caecilians ……
7. Name some importance of the Sagalla caecilian ……
8. What threats do you know that face the caecilians? ……..
9. Name ways in which you can do to protect or conserve the Sagalla caecilian? What other information do you know about the Sagalla caecilian? ……

C The Checklist

Institution (Respondent): …… **Date:** ……

The following are some of the major activities that can be done to promote the conservation of the Sagalla caecilian and its habitat: Which ones have you or your institution done?

1. Planting indigenous trees on communal/private lands.
2. Replacing exotic plants (e.g. Eucalyptus, pine) with indigenous trees.
3. Farm terracing and reinforcing with Napier grass or its equivalent, e.g. stone piles etc.
4. Planting more banana plants as a source of food and control of surface runoff.
5. Increased use of organic manure (cow-dug, compost, farm trash).
6. Not burning farm trash but letting it to decompose.
7. Educating others on the presence and importance of Sagalla caecilian in the environment.

ZOOLOGICAL RESEARCH

Interspecific variation of thermoregulation between small migratory and resident passerines in Wenzhou

Qing-Gang QIAO[1], Hong-Ji LIANG[1], Min-Lan BAI[1], Wei-Hong ZHENG[1,2], Jin-Song LIU[1,2,*]

[1] *School of Life and Environmental Sciences*, *Wenzhou University, Wenzhou* 325035, *China*

[2] *Zhejiang Provincial Key Lab for Subtropical Water Environment and Marine Biological Resources Protection*, *Wenzhou* 325035, *China*

ABSTRACT

Physiological adaptation arises from several fundamental sources of phenotypic variation. Most analyses of metabolic adaptation in birds have focused on the basal metabolic rate (BMR), the lower limit of avian metabolic heat production. In this study, we investigated thermoregulation in three passerine species; the yellow-billed grosbeak *Eophona migratoria*, white-rumped munia *Lonchura striata* and black-throated bushtit *Aegithalos concinnus*, in Wenzhou, China. Metabolic rate was measured using the closed-circuit respirometer containing 3.5 L animal chambers. Body temperature (T_b) was measured during metabolic measurements using a lubricated thermocouple. The minimum thermal conductance of these species was calculated by measuring their T_b and metabolic rates. The yellow-billed grosbeak remained largely normothermic, and the white-rumped munia and black-throated bushtit exhibited variable T_b at ambient temperatures (T_a). Mean metabolic rates within thermal neutral zone were 2.48±0.09 O_2 (mL)/g/h for yellow-billed grosbeaks, 3.44±0.16 O_2 (mL)/g/h for white-rumped munias, and 3.55±0.20 O_2 (mL)/g/h for black-throated bushtits, respectively. Minimum thermal conductance of yellow-billed grosbeak, white-rumped munia and black-throated bushtit were 0.13±0.00, 0.36±0.01, and 0.37±0.01 O_2 (mL)/g/h/˚C, respectively. The ecophysiological characteristics of these species were: (1) the yellowbilled grosbeak had relatively high T_b and BMR, a low lower critical temperature and thermal conductance, and a metabolic rate that was relatively insensitive to variation in T_a; all of which are typical of cold adapted species and explain its broader geographic distribution; (2) the white-rumped munia and black-throated bushtit had high thermal conductance, lower critical temperature, and relatively low BMR, all which are adapted to warm environments where there is little selection pressure for metabolic thermogenesis. Taken together, these data illustrate small migratory and resident passerines that exhibit the different characteristics of thermoregulation.

Keywords: Basal metabolic rate; Body temperature; Thermal conductance; *Eophona migratoria*; *Lonchura striata*; *Aegithalos concinnus*

INTRODUCTION

Heat production in response to ambient temperature is commonly referred to as facultative, or adaptive, thermogenesis (Angilletta et al., 2010; Silva, 2006; Zhou et al., 2016). Endotherms primarily use mechanisms that equalize rates of heat production and loss to maintain a high and constant body temperature (Corp et al., 1997; Liu et al., 2004a). The necessity of maintaining an optimal body temperature is one of the major factors influencing the abundance and distribution of birds (Liu et al., 2005; Weathers, 1979), and birds have evolved many morphological and physiological adaptations to achieve this (Swanson & Merkord, 2013; Wiersma et al., 2007). Physiological adaptation arises from several fundamental sources of phenotypic variation. Most analyses of metabolic adaptation in birds have focused on the basal metabolic rate (BMR), the lower limit of avian metabolic heat production (McKechnie et al., 2006; McNab, 2009). BMR is a standardized baseline metabolic parameter that reflects a species' resting energy requirements in the absence of the increased metabolic demands associated with thermoregulation, digestion, activity or circadian rhythms (McKechnie, 2008; McNab, 2009; Zhou et al., 2016). BMR is a widely-accepted benchmark of metabolic expenditure for birds that is commonly used as a measure of

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*Corresponding author, E-mail: ljs@wzu.edu.cn

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the energetic cost of thermoregulation. It has consequently been the focus of considerable research interest from environmental physiologists and comparative physiologists (e.g., Liu et al., 2005; Zheng et al., 2014a).

Ambient temperature (T_a) is considered one of the most important environmental factors affecting birds because it causes marked changes in their energy expenditure (Nzama et al., 2010) and has driven the evolution of a suite of morphological and physiological adaptations (Swanson et al., 2014). A considerable body of research has been conducted to examine the effects of temperature on animal adaptation, survival, and reproductive success (Sgueo et al., 2012; Zhou et al., 2016). In a comparative study of small birds with different habitats and habits, Rezende et al. (2002) recently emphasized the ecological significance of BMR. For example, tropical birds typically have a lower BMR than cold temperate birds, which is thought to be an adaptation to avoid heat stress and conserve water (Weathers, 1997; Wiersma et al., 2007). Conversely, the higher BMR of cold temperate and Arctic birds is thought to be an adaptation to colder temperatures and shorter breeding seasons (Klaassen, 1995; Zheng et al., 2014b). It has been suggested that the BMR of a number of long-distance migratory birds is lower in their tropical overwintering range than at their temperate breeding grounds (Lindström & Klaassen, 2003; Zheng et al., 2013). The higher metabolic capacities of high latitude species may involve a combination of genetic responses to climatic factors (Liknes & Swanson, 2011; Swanson, 2010; Wikelski et al., 2003). These findings indicate that environmental conditions are very important in shaping the thermoregulatory features of a species. There is now considerable evidence to show that the metabolic characteristics of birds are part of a network of physiological mechanisms that mediate major life-history trade-offs (Wikelski et al., 2003).

The yellow-billed grosbeak *Eophona migratoria* is a migratory bird that inhabits vast areas of northeast Asia. The white-rumped munia *Lonchura striata* is a common resident breeder in southern China and South Asia. The distribution of the black-throated bushtit *Aegithalos concinnus* ranges from the foothills of the Himalayas, across northern India through Nepal, to northern Vietnam and Taiwan (MacKinnon & Phillipps, 2000). Although all three species experience the same temperature and photoperiod in autumn and winter in Wenzhou, the yellow-billed grosbeak is a Palearctic bird that migrates to Wenzhou in winter whereas the white-rumped munia and black-throated bushtit are Indomalayan species that are resident in Wenzhou, and their thermoregulatory responses could differ (e.g., climatic differences across the ranges). To test the hypothesis that characteristics of thermoregulation in these three species are consistent with their respective biogeographic distributions, we compared body temperature, metabolic rate and thermal conductance among three small birds at different ambient temperatures. These results could make us better understand how these species adapt their environments. In addition, through the comparison with other small birds, it will help identify sources of variation in thermoregulatory characteristics in these small birds.

MATERIALS AND METHODS

Animals

Seven yellow-billed grosbeaks (six male, one female), ten white-rumped munias (six male, four female) and nine black-throated bushtits (seven male, two female) were captured by mist nets in Wenzhou city (N27°29', E120°51'), Zhejiang Province, China. The yellow-billed grosbeak *Eophona migratoria* is a granivorous and insectivorous migratory bird. The white-rumped munia *Lonchura striata* is a gregarious bird that feeds mainly on seeds. The black-throated bushtit *Aegithalos concinnus* mainly feeds on small insects and spiders, as well as small seeds, fruits and berries (particularly raspberries). The climate in Wenzhou is warm-temperate with the mean annual temperature is 18 ˚C. There are seven months of the year (March through September) in which the maximum temperature is above 37 ˚C (Zheng et al., 2008a; 2014a). All experiments were carried out from October to December 2012. Animals were kept in individual cages (50 cm×30 cm×20 cm) under natural photoperiod (14L:10D) with lights on at 0600h and temperature (25 ˚C). Food and water were supplied *ad libitum*. Yellow-billed grosbeaks and white-rumped munias were fed millet seeds, and black-throated bushtits were fed bird cake and mealworm *Tenebriomolitor* larvae. The mean body mass of yellow-billed grosbeaks, white-rumped munias and black-throated bushtits was 50.5±0.6 g (47.9-52.4 g), 12.6±0.3 g (11.1-14.5 g) and 6.8±0.1 g (6.4-7.4 g), respectively. All experimental procedures were approved by the Wenzhou City Animal Care and Use Committee, Zhejiang Province, China (Wu et al., 2015).

Measurement of metabolic rate

Oxygen consumption was measured using a closed-circuit respirometer according to the methods described by Górecki (1975) and Liu et al. (2004a; 2005). The volume of the metabolic chamber was 3.5 L and its temperature was controlled by a water bath in Artificial Climatic Engine (BIC-300, Shanghai) and maintained to ±0.5 ˚C. Every bird was tested only one Ta per day with at least two days between tests (Xia et al., 2013). Oxygen consumption rates were measured over a temperature range of 5 ˚C to 35 ˚C. Food was withheld four hours before animals were placed in the metabolic chamber to minimize the heat increment associated with feeding before each test. Birds were weighed to the nearest 0.1 g before being put in the chamber. Water and CO_2 were absorbed from the air in the chamber by silica gel and KOH. All measurements were made between 2000h and 2400h. Each trial lasted for one hour and commenced after animals had been inside the metabolic chamber for about one hour to acclimate. The reading interval for O_2 consumption was 10 min. Two or three consecutive, stable, minimum, recordings were used to calculate metabolic rates (Zheng et al., 2008b). Records of oxygen consumption when birds were active within the chamber were not used to compute the metabolic rate of each individual. Metabolic rates were expressed as O_2 (mL)/g/h, and corrected to standard

temperature and pressure conditions (Schmidt-Nielsen, 1997). Body mass was measured to the nearest 0.1 g before and after experiments. Mean body mass was used in calculations (Liu et al., 2005; Wu et al., 2015).

Measurement of body temperature

Body temperature (T_b) was measured during metabolic measurements using a lubricated thermocouple. This was inserted into the cloaca of each bird to a depth at which a slight withdrawal did not result in a change in the reading (1-2 cm). Thermocouple outputs were digitized using a thermocouple meter (Beijing Normal University Instruments Co., China) (Wu et al., 2015).

Calculation of thermal conductance

Total wet thermal conductance (C, O_2 (mL)/g/h/˚C) at any given T_a was calculated using the formula:

$$C=MR/(T_b-T_a) \quad (1)$$

Where MR is metabolic rate (O_2 (mL)/g/h/˚C), T_b the body temperature (˚C), and T_a the ambient temperature (˚C). This formula was suggested by Aschoff (1981) for calculating conductance at any given T_a.

Statistics

The data were analyzed using the SPSS statistical package (version 12.0 for windows). The effect of T_a on body temperature, metabolic rate and thermal conductance were analyzed using repeated measures ANOVA. Where appropriate, multiple *post hoc* comparisons were performed using the least significant difference method (LSD). The relationships between metabolic rate and T_b and T_a were modeled by fitting linear regression models. The relationships between thermal conductance and T_a were modeled by fitting exponential equation models, to the data, as appropriate. All results were expressed as mean±*SE* and $P<0.05$ was taken to be statistically significant.

RESULTS

Males and females of each species did not differ significantly in any measured variable ($P>0.05$ in all cases), so we pooled data for each species for subsequent analyses.

Yellow-billed grosbeak

The mean T_b of this species was 39.9±0.1 ˚C. Although there was no significant difference in T_b over a range of T_a from 5 ˚C to 32.5 ˚C ($F_{7,41}=1.059$, $P>0.05$, Figure 1A), there was, however, significant differences in metabolic rate (MR) over this temperature range ($F_{7,41}=21.231$, $P<0.001$, Figure 1B). We were unsuccessful in identifying a join-point using a two-phase regression procedure (Nickerson et al., 1989), so we instead fit a linear regression model to data below 25 ˚C. Below 25 ˚C, MR increased with decreasing temperature as per the following equation:

MR (O_2 (mL)/g/h)= 5.07-0.11T_a ($P<0.001$, $R^2=0.817$, $n=31$) (2)

At 25 ˚C, MR appeared independent of T_a, averaging 2.48±0.09 O_2 (mL)/g/h. The line described by the above equation intersected MR at 23.5 ˚C, the lower critical temperature. Thermal conductance increased from 15 °C to 32.5°C ($F_{7,41}=49.802$, $P<0.001$, Figure 1C), as it was stable within the range of 5˚ C-15 °C. Minimum thermal conductance was 0.13± 0.00 O_2 (mL)/g/h/˚C (Table 1). Thermal conductance increased exponentially from 25 ˚C to 32.5 ˚C as per the equation:

$$\text{Log } C \text{ } (O_2 \text{ (mL)/g/h/˚C})=-2.00+0.05\ T_a \quad (P<0.001,\ R^2=0.746,\ n=22) \quad (3)$$

Maximum thermal conductance was 0.41±0.02 O_2 (mL)/g/h/˚C at 32.5 ˚C.

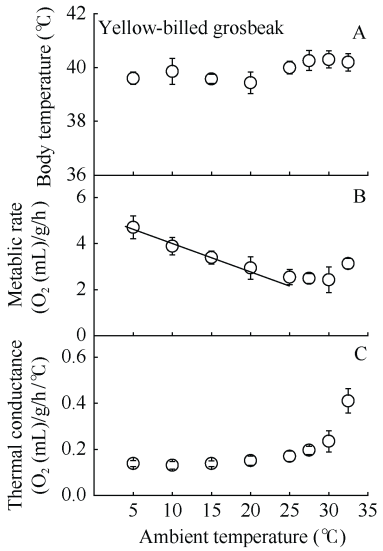

Figure 1 Mean body temperature (A), metabolic rate (B) and thermal conductance (C) of wild caught yellow-billed grosbeaks *Eophona migratoria* measured in an experimental facility in Wenzhou, China at ambient temperatures of approximately 5 ˚C-32.5 ˚C

White-rumped munia

The T_b of the white-rumped munia fluctuated significantly over ambient temperatures between 5 ˚C and 37.5 ˚C ($F_{9,90}=11.190$, $P<0.001$, Figure 2A). Mean T_b was 39.9±0.1 ˚C (Table 1) and ranged from 39.1±0.2 ˚C at 5 ˚C to 40.6±0.2 ˚C at 37.5 ˚C. There was a positive, linear relationship between T_b and T_a over this temperature range as per the following equation:

$$T_b=38.96+0.04\ T_a \quad (P<0.001,\ R^2=0.511,\ n=100) \quad (4)$$

The MR of this species varied significantly between 5 ˚C and 37.5 ˚C ($F_{9,90}=98.310$, $P<0.001$, Figure 2B). Between 30 ˚C and 35 ˚C, MR appeared to be independent of Ta and averaged 3.44±0.16 O_2 (mL)/g/h ($n=36$). For Tas below 30 ˚C,

$$MR \text{ } (O_2 \text{ (mL)/g/h})=13.09-0.28\ Ta \quad (P<0.001,\ R^2=0.824,\ n=70) \quad (5)$$

The line intersected MR at 34.5 ˚C, so the lower critical temperature was 34.5 ˚C. Thermal conductance varied significantly within a temperature range from 5 ˚C to 37.5 ˚C ($F_{9,90}=51.434$, $P<0.001$, Figure 2C), but was stable within a temperature range of 5 ˚C to 20˚C. Minimum thermal conductance was 0.36±0.01 O_2 (mL)/g/h/˚C. There was a

Table 1 Energetic parameters of wild-caught yellow-billed grosbeaks, white-rumped munias and black-throated bushtits measured in an experimental facility at Wenzhou, China

	Yellow-billed grosbeak	White-rumped munia	Black-throated bushtit
Body mass (g)	50.5±0.6	12.6±0.3	6.8±0.1
Body temperature (˚C)	39.9±0.1	39.9±0.1	38.9±0.1
BMR (O_2 (mL)/g/h)	2.48±0.09	3.44±0.16	3.55±0.20
Expectation ratio (% predicted)	172	124	99
a (O_2 (mL)/g/h)	5.05	12.59	14.20
b (O_2 (mL)/g/h/˚C)	-0.14	-0.24	-0.37
R^2	0.799	0.792	0.839
P	<0.001	<0.001	<0.001
T_{lc} (˚C)	23.5	34.5	28.8
Conductance (O_2 (mL)/g/h/˚C)	0.13±0.00	0.36±0.01	0.37±0.01
Expectation ratio (% predicted)	137	200	155

The equations are in the form of MR (O_2 (mL)/g/h)=a+b×T_a. Values are mean±SE. BMR is the basal metabolic rate, T_{lc} is the lower critical temperature. The expectationratio of BMR and conductance were predicted from the appropriate equation in Londoño et al. (2015) or Aschoff (1981), respectively. % predicted=(observed/predicted)×100.

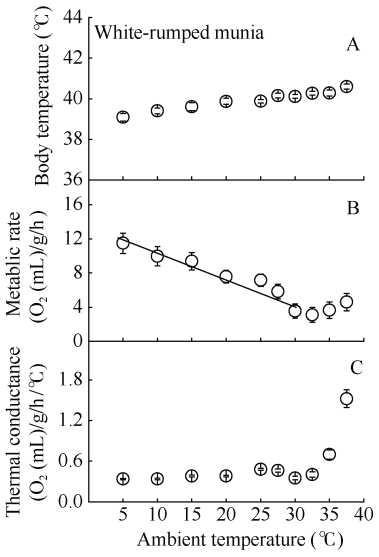

Figure 2 Mean body temperature (A), metabolic rate (B) and thermal conductance (C) of wild caught white-rumped munias *Lonchura striata* measured in an experimental facility in Wenzhou, China at ambient temperatures of approximately 5 ˚C-37.5 ˚C

significant, linear relationship between thermal conductance and T_a between 32.5 ˚C and 37.5˚C described by the equation:

Log C (O_2 (mL)/g/h/˚C)=-4.16+0.15 T_a (P<0.001, R^2=0.760, n=30) (6)

Maximum thermal conductance attained 1.52±0.13 O_2 (mL)/g/h/˚C at 37.5 ˚C.

Black–throated bushtit

T_b varied significantly in this species between 5 ˚C and 34 ˚C ($F_{7,64}$=8.173, P<0.001, Figure3A). Mean T_b was 38.9±0.1 ˚C (Table 1) and tended to increase with T_a, ranging from 37.8±0.4 ˚C at 5 ˚C to 40.4±0.3 ˚C at 34 ˚C. The relationship between T_b and T_a over this temperature range can be described by the following equation:

T_b=37.36+0.08 T_a (P<0.001, R^2=0.362, n=72) (7)

There were also significant differences in MR from 5 ˚C to 34˚C ($F_{7,64}$=58.547, P<0.001, Figure3B). At 28 ˚C, MR appeared to be independent of T_a and averaged 3.55±0.20 O_2 (mL)/g/h. For T_as below 28˚C,

MR (O_2 (mL)/g/h)=14.20-0.37 T_a (P<0.001, R^2=0.839, n=54) (8)

The line intersected MR at 28.8 ˚C, so the lower critical temperature was 28.8 ˚C. Thermal conductance increased significantly with T_a ($F_{7,64}$=20.001, P<0.001, Figure3C). Minimum thermal conductance was 0.37±0.00 O_2 (mL)/g/h/˚C from 5 ˚C to 28 ˚C. The relationship between thermal conductance and T_a can be described by the equation:

Log C (O_2 (mL)/g/h/˚C)=-2.40+0.07 T_a (P<0.001, R^2=0.643, n=27) (9)

Maximum thermal conductance averaged 0.80±0.08 O_2 (mL)/g/h/˚C at 34 ˚C.

DISCUSSION

Interspecific variation in metabolic rate, thermal neutral zone (TNZ) and thermoregulation

Londoño et al. (2015) used allometric equations to calculate the expected the BMR of a range of bird species from their published body mass. According to Londoño et al. (2015), the

relationship between BMR and M_b for tropical species, BMR (watts)=0.449 $M_b^{0.589}$ (*BMR*=Watt, M_b=g); for temperate species, BMR (watts)=0.023 $M_b^{0.729}$. The BMRs of the yellow-billed grosbeak, white-rumped munia and black-throated bushtit were 172%, 124% and 99%, respectively, of the values predicted from body mass (Londoño et al., 2015). At lower T_a values, the BMRs of the yellow-billed grosbeak, white-rumped munia and black-throated bushtit generally decreased with increasing temperature, a pattern typical of endotherms (Schmidt-Nielsen, 1997; Willmer et al., 2005). Consequently we consider the minimum *MR* recorded in these species to be their true BMR. Many factors, such as body size, phylogeny, climate conditions, activity, and feeding habits, are thought to affect the metabolic levels of birds (McNab, 2000; 2009). Rezende et al. (2002) and McNab (2009) suggested that avian BMR is generally correlated with climate. A reduced level of endogenous heat production may thus be adaptive in low-latitude species (Wiersma et al., 2007; Wikelski et al., 2003), and conversely, higher metabolic rates may be adaptive in mid-latitude and high-latitude species (Swanson, 2010; Zheng et al., 2008b; 2014b). The low BMR of tropical species may arise directly from living in warm environments, with modest demands for metabolic thermogenesis and activity reflected in low rates of baseline energy expenditure (Jetz et al., 2008; White et al., 2007), and linked to their generally slow pace of life and lower investment in reproduction (Londoño et al., 2015; Ricklefs & Wikelski, 2002; Wiersma et al., 2007; Williams et al. 2010). Conversely, the higher BMR of temperate and arctic birds is thought to be an adaptation to colder temperatures and higher investment in reproduction (Klaassen, 1995; Londoño et al., 2015). Previously published data on the BMR of temperate and tropical birds, together with metabolic rates predicted from Londoño et al. (2015) equation are presented (Table 2). The BMR of temperate species is generally higher tropical species.

Table 2 Comparison of observed and predicted basal metabolic rates (BMR) of bird species from cold, temperate, and tropical regions

Species	Body mass (g)	BMR (Watt)	Expectation ratio (%)	Reference
Cold and temperate region				
Black-capped chickadee *Parus atricapillus*	13.8	0.35	223	1
Rufous-necked snowfinch *Montifringilla ruficollis*	22.8	0.47	211	2
Eurasian skylark *Alauda arvensi*	32.0	0.73	255	3
Woodlark *Lullula arborea*	25.6	0.58	237	3
Chestnut bunting *Emberiza rutila*	15.3	0.34	203	4
Little bunting *Emberiza pusilla*	11.3	0.26	196	4
Scarlet rosefinch *Carpodacus erythrinus*	24.2	0.57	242	5
Pallas's rosy finche *Carpodacus roseus*	22.5	0.54	242	6
Brambling *Fringilla montifringilla*	18.0	0.42	222	6
Common redpoll *Acanthis flammea*	11.6	0.29	210	6
Siberian accentor *Prunella montanella*	13.5	0.32	210	7
Chestnut-flanked white-eye *Zosterops erythropleura*	9.2	0.24	207	8
Yellow-browed bunting *Emberiza chrysophrys*	15.9	0.32	187	8
Black-faced bunting *Emberiza spodocephala*	15.1	0.40	239	9
Bohemian waxwing *Bombycilla garrulus*	64.9	0.84	175	9
Eurasian oystercatcher *Haematopus ostralegus*	554.0	2.91	127	10
Black-bellied plover *Pluvialis squatarola*	226.0	1.78	149	10
Ruddy turnstone *Arenaria interpres*	90.0	0.92	150	10
Little ringed plover *Charadrius dubius*	36.0	0.42	134	11
Common quail *Coturnix coturnix*	97.0	0.89	138	11
Eurasian sparrow hawk *Accipiter nisus*	135.0	0.95	116	11
King quail *Excalfactoria chinensis*	42.4	0.42	119	12
Barn owl *Tyto alba*	456.1	2.61	131	12
Western screech owl *Megascops kennicottii*	147.3	1.27	145	12
Rock parrot *Neophema petrophila*	48.4	0.63	162	13
Yellow-billed grosbeak *Eophona migratoria*	48.7	0.67	172	This Study

Continued

Species	Body mass (g)	BMR (Watt)	Expectation ratio (%)	Reference
Tropical				
Dusky munia *Lonchura fuscans*	9.5	0.10	60	14
Golden-collared manakin *Manacus vitellinus*	15.5	0.23	105	15
Red-capped manakin *Pipra mentalis*	12.3	0.19	101	15
Orange-cheeked waxbill *Estrilda melpoda*	7.5	0.13	90	16
Zebra finch *Poephila guttata*	12.1	0.18	96	16
Gouldian finch *Chloebia gouldiae*	15.5	0.22	98	16
Cut-throat finch *Amadina fasciata*	17.2	0.21	91	16
Java sparrow *Padda oryzivora*	25.4	0.31	104	16
Gouldian finch *Chloebia gouldiae*	17.1	0.23	99	17
Crested myna *Acridotheres cristatellus*	117.7	0.70	96	18
Puerto Rican tody *Todus mexicanus*	6.1	0.11	86	13
American pygmy kingfisher *Chloroceryle aenea*	11.8	0.17	90	11
Chestnut-backed antbird *Myrmeciza exsul*	28.3	0.29	92	11
Blue-crowned motmot *Momotus momota*	123.1	0.52	69	12
Andean cock-of-the-rock *Rupicola peruvianus*	246.5	1.08	96	12
White-tufted sunbeam *Aglaeactis castelnaudii*	6.9	0.10	73	12
Great sapphirewing *Pterophanes cyanopterus*	10.8	0.11	62	12
White-bellied woodstar *Chaetocercus mulsant*	3.6	0.07	75	12
Chestnut-capped puffbird *Bucco macrodactylus*	23.7	0.18	63	12
Semicollared puffbird *Malacoptila semicincta*	47.1	0.33	78	12
Black-fronted nunbird *Monasa nigrifrons*	86.0	0.46	76	12
White-fronted nunbird *Monasa morphoeus*	67.5	0.45	86	12
Rufous-breasted piculet *Picumnus rufiventris*	21.9	0.26	96	19
White-rumped munia *Lonchura striata*	12.6	0.24	124	This study
Black-throated bushtit *Aegithalos concinnus*	6.8	0.13	99	This study

Basal metabolic rates (BMR) was predicted by the appropriate equation in Londoño et al. (2015). % predicted=(observed/ predicted)×100. For each species, body mass, BMR and expectation ratio (%) are provided. References: 1 Chaplin, 1974; 2 Deng & Zhang, 1990; 3 Tieleman et al., 2002; 4 Liu et al., 2001a; 5 Liu et al., 2001b; 6 Liu et al., 2004a; 7 Liu et al., 2004b; 8 Liu et al., 2005; 9 Li et al., 2005; 10 McKechnie & Wolf, 2004; 11 Wiersma et al., 2007; 12 Londoño et al., 2015; 13 White et al., 2007; 14 Weathers, 1977; 15 Bartholomew et al., 1983; 16 Marschall & Prinzinger, 1991; 17 Burton & Weathers, 2003; 18 Lin et al., 2010.

Feeding habits are also an important factor affecting both the metabolic rates of animals and their geographic distribution. Birds feeding on seeds and fruits tend to have high metabolic rates that thought to be related to the consistency and abundance of food in their environment. In contrast, insectivorous birds tend to have lower metabolic rates (McNab, 1988). The yellow-billed grosbeak and white-rumped munia mainly feed on seeds with the addition of some insects and fruit in summer and autumn, whereas the black-throated bushtit is predominantly insectivorous. It is possible that the different dietary preferences of these species may affect their respective metabolic rates. Our data show that the BMR of the yellow-billed grosbeak and white-rumped munia were higher than the predicted BMR using allometric equations, whereas that of the black-throated bushtit was similar to predicted values, and that this difference could be due to the grosbeak and munia being predominantly granivorous, whereas the bushtit is more insectivorous. These results are consistent with the previous studies (McNab, 1988).

TNZ is defined as the range of T_a at which temperature regulation is achieved only by control of sensible heat loss, without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 1987; Willmer et al., 2005). A lower critical temperature and broader TNZ are typical of species that are adapted to cold (Schmidt-Nielsen, 1997; Willmer et al., 2005). For example, the arctic ptarmigan *Lagopus spp.* does not increase its metabolic rate unless the external temperature falls to -5 °C, and even then its metabolic rate is not greatly affected by ambient temperature (Mortensen & Blix, 1986). Conversely, a higher upper critical

temperature is typical of adaptation to hot climates, especially with regard to water conservation (Williams & Tieleman, 2000). For example, the Chinese hwamei (*Garrulaxcanorus*) may increase its metabolic rate if the ambient temperature drops to just 31 °C, and its metabolic rate is much more sensitive to change in ambient temperature (Wu et al., 2015; Xia et al., 2013). The lower critical temperature of the yellow-billed grosbeak was 23.5 ˚C, and the slope of the regression equation describing the relationship between metabolic rate and ambient temperature for this species was 0.14 (Table 1), results typical of a cold tolerant species. However, the white-rumped munia and black-throated bushtit increased their metabolic rates when the ambient temperature dropped to 30 °C and 28 °C, respectively, and the slopes of the regression equations describing the relationship between metabolic rate and temperature in these species were steeper; 0.24 O_2 (mL)/g/h/˚C for the white-rumped munia and 0.37 O_2 (mL)/g/h/˚C for black-throated bushtit (Table 1). These data indicate that these species are relatively intolerant to cold.

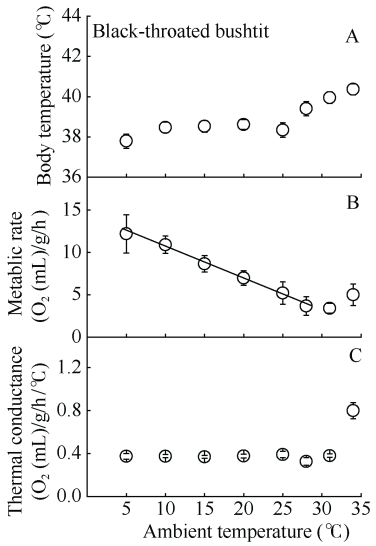

Figure 3 Mean body temperature (A), metabolic rate (B) and thermal conductance (C) of wild-caught black-throated bushtits *Aegithalos concinnus* measured in an experimental facility in Wenzhou, China at ambient temperatures of approximately 5 ˚C-34 ˚C

Interspecific variation in body temperature and thermal conductance

Considerable research has been devoted to the study of avian energetics, including body temperature (Clarke & Rothery, 2008; Prinzinger et al., 1991; Xia et al., 2013). The body temperature of birds depends upon their metabolic rate and heat loss. Small birds have higher body temperatures than larger ones because they have higher mass-specific rates of heat production. For example, the rest-phase T_b of most birds is 38.4 ˚C or less, but that of passerines is 39.0 ˚C (Prinzinger et al., 1991). We found that the yellow-billed grosbeak had the least variable T_b among the three species studied at lower T_a values. The high and constant T_b of the yellow-billed grosbeak would be advantageous in boreal latitudes, whereas the lower T_b of the white-rumped munia and black-throated bushtit may be the result of an optimization process through which these species attempt to minimize their energy expenditure (McKechnie & Lovegrove, 2001).

According to Aschoff (1981) formula, minimal thermal conductance depends on body temperature, metabolic rate and body mass. Small birds have a relatively large surface to volume ratio, less insulation, and higher heat loss, resulting in higher thermal conductance (Schmidt-Nielsen, 1997). Our results show that the minimum thermal conductance of the yellow-billed grosbeak, white-rumped munia and black-throated bushtit were 137%, 200% and 155%, respectively, of the values predicted from Aschoff (1981) allometric equation, indicating that these species are poorly insulated for their body size. Birds that live at low latitudes generally have higher thermal conductance than expected based on their body mass, a feature that may facilitate heat loss (Schleucher & Withers, 2001). In Wenzhou, the mean temperature in October is about 23 °C, so birds inhabiting this region would be expected to have low metabolic heat production and high thermal conductance as a means of avoiding hyperthermia (Liu et al., 2006; Xia et al., 2013; Wu et al., 2015).

Metabolic properties and distribution

The metabolic properties of these species may also be an important factor affecting their distribution in China. In the present study, the high BMR, low lower critical temperature, and relatively stable metabolic rate, of the yellow-billed grosbeak are typical of a species adapted to cold. These features, in conjunction with its relatively lower thermal conductance and granivorous diet, may explain its broad geographic distribution. Conversely, the relatively low BMR, high thermal conductance, and temperature-sensitive metabolic rate, of the black-throated bushtit are typical of a tropical species. These features, together with its predominantly insectivorous diet, may explain why it is confined to relatively warm regions where insects and other invertebrates are more abundant. The white-rumped munia shares characteristics of both the yellow-billed grosbeak and black-throated bushtit. It has a relatively high BMR, thermal conductance, lower critical temperature, and its metabolic rate is relatively sensitive to changes in ambient temperature. These features, coupled with its food habit, may explain its relatively warm geographic distribution. In the present study, our data illustrate variation in the thermoregulatory characteristics of small passerine species that differ in their biogeographic distributions.

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Morphology and molecular phylogeny of two colepid species from China, *Coleps amphacanthus* Ehrenberg, 1833 and *Levicoleps biwae jejuensis* Chen et al., 2016 (Ciliophora, Prostomatida)

Bo-Rong LU[1], Ming-Zhen MA[1], Feng GAO[2,*], Yu-Hong SHI[1], Xiang-Rui CHEN[1,*]

[1] *School of Marine Sciences*, *Ningbo University*, *Ningbo* 315211, *China*

[2] *Institute of Evolution & Marine Biodiversity*, *Ocean University of China*, *Qingdao* 266003, *China*

ABSTRACT

Two colepid ciliates, *Coleps amphacanthus* Ehrenberg, 1833 and *Levicoleps biwae jejuensis* Chen et al., 2016, were first recorded in China. Their living morphology, infraciliature and small subunit (SSU) rRNA gene sequences were determined using standard methods. The improved diagnosis of *Coleps amphacanthus* is as follows: cell size about 100× 50 μm *in vivo*, barrel-shaped; 22-28 ciliary rows each composed of about 14-21 monokinetids and two perioral dikinetids; 5-10 caudal cilia; and one terminal contractile vacuole. *Levicoleps biwae jejuensis* was also investigated, with an improved diagnosis given based on previous and present work. The phylogenetic analyses based on SSU rRNA gene sequences revealed that all *Coleps* species were grouped together, except for *Coleps amphacanthus*, which was grouped into a clade of the genus *Levicoleps*.

Keywords: Ciliate; *Coleps*; *Levicoleps*; New record; Phylogeny; SSU rRNA; Taxonomy

INTRODUCTION

Colepid ciliates are commonly found in a wide range of habitats, including benthic, pelagic and marine psammobiotic. They are one of the main components of the microbial community and play an important role in the function of the microbial food webs (Finlay & Fenchel, 1996; Song et al., 2009). Since the first species, *Coleps hirtus* (Müller, 1786) Nitzsch, 1827, was reported two centuries ago, more than 40 species of this family have been found and recorded. They are characterized by cylindrical or barrel-shaped bodies covered by unique calcified cuirasses (armored plates with small lateral plate processes arranged in longitudinal rows), sometimes with anterior and/or posterior spines. In general, their caudal cilia are clearly longer than other somatic cilia (Corliss, 1979; Dragesco & Dragesco-Kernéis, 1991; Foissner, 1983; Kahl, 1930; Noland, 1925; Obolkina, 1995). Individual taxa may be characterized by a variety of morphological features including the number of armor tiers, structure of plates, presence or absence of spines, and number of adoral organelles (Foissner et al., 2008). According to these taxonomic standards, some new genera have been established in recent years, including the construction of *Levicoleps* due to the absence of spines and armor tiers with *hirtus*-type plates and *Kotinia* characterized by spiny armor composed of eight tiers and five adoral organelles (Chen et al., 2009, 2010, 2012, 2016; Foissner et al., 2008; Obolkina, 1995).

The present paper provides a morphological description of a poorly known species, *Coleps amphacanthus* Ehrenberg, 1833, and a new record of *Levicoleps biwae jejuensis* Chen et al., 2016, in China. Phylogenetic analyses based on SSU rRNA gene sequences were also performed.

MATERIALS AND METHODS

Sample collection, observation, and identification

Coleps amphacanthus Ehrenberg, 1833 was collected from the brackish water of Hangzhou Bay, Ningbo (N30°22′; E121°12′), China, on 22 July, 2014. The water temperature was about 25°C and salinity was about 2‰. Samples with decaying plants were collected from the surface of the sediment using a plastic dropper, then diluted with untreated water from the collection site.

Levicoleps biwae jejuensis Chen et al., 2016 was collected on 22 June, 2015, from a freshwater pond in Ningbo (N29°55′;

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*Corresponding authors, E-mail: gaof@ouc.edu.cn;xiangruichen@126.com

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E121°39′). The water temperature was about 23 °C. Samples were collected by 20 μm mesh plankton nets from the upper layer of the water.

The behavior of the organisms was studied in Petri dishes under a dissecting microscope. Living morphology was investigated under a compound microscope equipped with a high-power oil immersion objective as well as differential interference contrast optics (Olympus BH-2, Olympus Optical Co., Ltd, Tokyo, Japan). The infraciliature was revealed via protargol impregnation (Wilbert, 1975). Counts and measurements of the morphological characteristics of stained specimens were performed at a magnification of ×1 250. Drawings were made with the help of a camera lucida. Terminology used is primarily in accordance with Foissner et al. (2008) and Chen et al. (2010).

DNA extraction, gene amplification, and sequencing

Genomic DNA was extracted from cells using the Dneasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The PCR amplification of the SSU rRNA gene sequences was performed using universal eukaryotic primers 18S-F (5'-AACCTGGTTGATCCTGCCAGT-3') and 18S-R (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al., 1988). PCR products were purified using an EasyPure®Quik Gel Extraction Kit (TransGen, EG101, Beijing, China), then cloned using a pEASY®-T1 Cloning kit (TransGen, CT101, Beijing, China). Sequencing was performed bidirectionally on an ABI 3700 sequencer (GENEWIZ Biotechnology Co., Ltd., Beijing, China).

Phylogenetic analyses

The two newly characterized SSU rRNA gene sequences and the sequences of another 45 species/populations obtained from the NCBI GenBank database were used for phylogenetic analyses. Sequences were aligned using Bioedit v7.1.3.0. (Hall, 1999) with the ClustalW algorithm. The resulting alignments were manually refined by trimming both ends. Final alignment included 1 767 characters and 47 taxa. *Paramecium tetraurelia*, *Ichthyophthirius multifiliis*, and *Tetrahymena pyriformis* were selected as outgroup taxa for phylogenetic analyses. Bayesian inference (BI) and maximum likelihood (ML) analyses were performed online using the CIPRES Science Gateway v3.3 (http: //www.phylo.org/portal2). BI analysis was performed with MrBayes on XSEDE v3.2.6 (Ronquist & Huelsenbeck, 2003) using the GTR+I+G model as selected by MrModeltest v2.2 (Nylander, 2004). The chain length of the Bayesian analyses was 10 000 000 generations, sampled every 100 generations. The first 10 000 sampled trees were considered as burn-in. ML analysis was performed with RAxML-HPC2 on XSEDE v8.2.4 (Stamatakis et al., 2008) using the GTR+I+G model as selected by Modeltest v3.4 (Posada & Crandall, 1998). The reliability of ML internal branches was assessed using a nonparametric bootstrap method with 1 000 replicates. MEGA v5.0 (Tamura et al., 2011) was used to view and edit tree topologies. Systematic classification mainly followed Lynn (2008) and Yi et al. (2010).

Hypothesis testing

To test the hypothesis that the morphologically-defined genera are monophyletic groups, RAxML (Shimodaira, 2002; Stamatakis et al., 2008) was used to generate ML trees with enforced topological constraints. For all constraints, internal relationships within the constrained groups and among the remaining taxa were unspecified. The site-wise likelihoods for the resulting constrained topologies and the non-constrained ML topologies were calculated using PAUP (Swofford, 2002). Approximately unbiased (AU) tests (Shimodaira, 2002) were performed using CONSEL v 0.1 (Shimodaira & Hasegawa, 2001) to obtain *P*-values.

RESULTS

***Coleps amphacanthus* Ehrenberg, 1833 (Table 1; Figure 1 and Figure 2)**

Coleps amphacanthus is a poorly known and rarely reported species (Foissner & O'Donoghue, 1990; Huttenlauch, 1986, 1987; Huttenlauch & Bardele, 1987; Kreutz & Foissner, 2006; Noland, 1925). According to these historic studies, the morphological features are not particularly stable and most descriptions lack the infraciliature. Therefore, re-description is necessary to reduce confusion and clarify intraspecific variations.

Improved diagnosis. Cells about (80-110) μm×(40-60) μm *in vivo*, barrel shaped. On average 22-28 ciliary rows, each composed of about 14-21 monokinetids and two perioral dikinetids. Anterior and posterior main plates each with 5-8 and 5-6 windows, respectively; anterior and posterior secondary plates with 2-3 windows, respectively. Five to ten caudal cilia. One subterminal contractile vacuole. Fresh- or brackish-habitat.

Voucher slide. A voucher slide with protargol-impregnated specimens was deposited in the Laboratory of Protozoology, Ocean University of China (registration number: LU-20140722-04-01).

Morphological description based on Chinese population. Body size (90-110) μm×(40-60) μm *in vivo*, usually about 100 μm×50 μm, barrel-shaped, with a slight wave-like arch at each window caused by sieve domes, usually slightly narrowed in the mid-body where the main armor plates abut. Ratio of body length to width about 2: 1. Anterior end broad, transversely truncated and crown-like due to triangle-shaped secondary tier plates; posterior end moderately rounded (Figure 1A; Figure 2A). Macronucleus spherical to broadly ellipsoidal with an average length: width ratio of 1.3, usually located at the equatorial level and near the periphery of the cell. Micronucleus globular and approximately 2 μm in diameter, adherent to the macronucleus (Figure 1D; Figure 2I, J). Contractile vacuole about 12 μm in diameter, positioned near the subterminal of the body (Figure 1A; Figure 2A). Cytoplasm colorless, usually containing several food vacuoles (10-20 μm in diameter) and mass of refractive lipid droplets (1-4 μm across) (Figure 1A; Figure 2A).

Armor composed of *hirtus*-type plates arranged in six tiers: circumoral, anterior secondary, anterior main, posterior main, posterior secondary, and caudal, with each tier consisting of about 25 rectangular plates (Figure 1A, B; Figure 2A). Circumoral tiers hardly recognizable *in vivo*. Anterior secondary plates about 12 μm in length, with two windows, two ciliary outlets,

Table 1 Morphometric data on *Coleps amphacanthus* (first line) and *Levicoleps biwae jejuensis* (second line)

Characters	*Min*	*Max*	*M*	*Mean*	*SD*	*SE*	*CV*	*n*
Body length	82	98	90.0	89.0	4.9	2.0	5.5	8
	85	103	94.0	95.1	5.2	1.3	5.5	15
Body width	39	51	45.0	46.0	5.0	2.0	10.1	8
	37	57	47.0	45.7	6.6	1.7	14.4	15
Somatic kineties, number	24	26	25.0	25.0	0.8	0.0	3.2	8
	21	24	22.5	23.1	0.8	0.2	3.5	15
Transverse ciliary rows, number*	18	18	18.0	18.0	0.0	0.0	0.0	9
	15	15	15.0	15.0	0.0	0.0	0.0	15
Macronucleus length	25	32	28.5	29.3	2.6	0.9	8.9	8
	21	38	29.5	27.7	4.8	1.2	17.3	15
Macronucleus width	16	29	22.5	23.8	4.5	1.6	18.9	8
	17	32	24.5	21.2	3.9	1.0	18.4	15

Data based on protargol-impregnated specimens. All measurements in μm. *CV*: coefficient of variation in %, *Max*: maximum, *Mean*: arithmetic mean, *M*: median, *Min*: minimum, *n*: number of specimens analysed, *SD*: standard deviation, *SE*: standard error. *: Excluding perioral dikinetids.

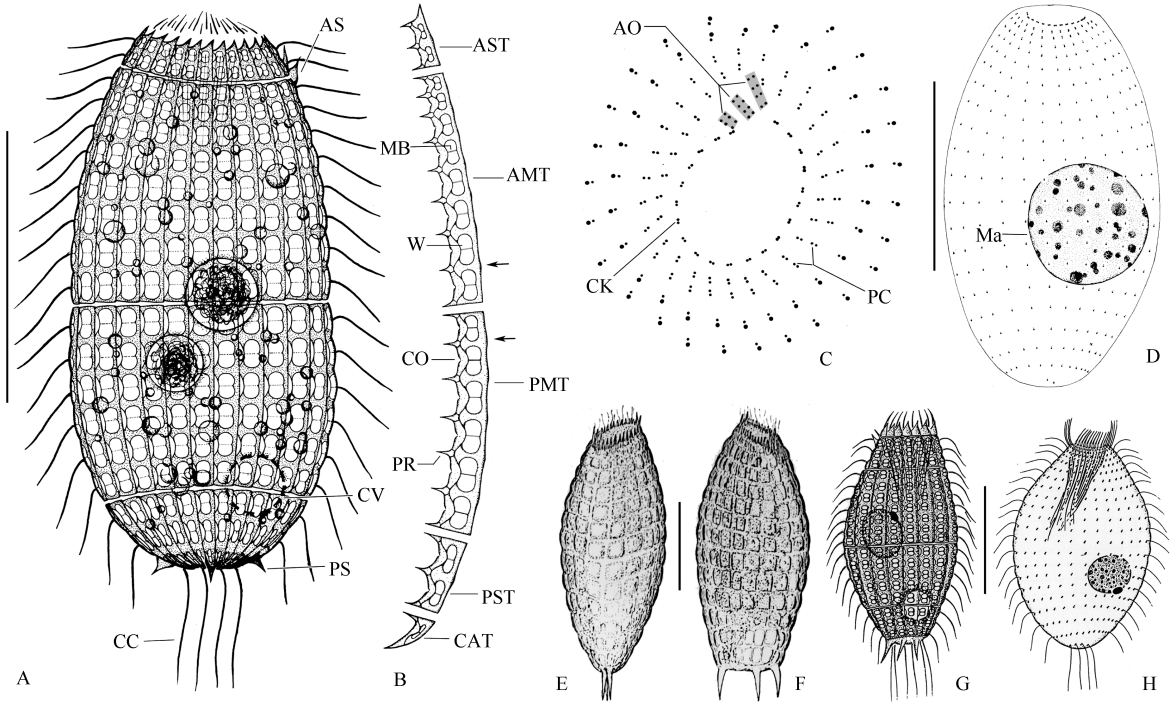

Figure 1 Morphology and infraciliature of *Coleps amphacanthus*

A: Lateral view shows body shape, smallish spines and characters of plates. B: Detailed structure of row of plates. C: Apical view of infraciliature, showing the ciliary pattern of the top end. D: Lateral view of the infraciliature. E, F: Lateral views of *Coleps amphacanthus* (from Noland 1925). G, H: Lateral views of living individual (G) and infraciliature (H) (from Foissner & O'Donoghue, 1990). Abbreviations: AO, adoral organelle; AMT, anterior main tier; AS, anterior spine; AST, anterior secondary tier; CAT, caudal tier; CC, caudal cilia; CK, circumoral kinety; CO, ciliary outlet; CV, contractile vacuole; Ma, macronucleus; MB, midbar; PC, perioral ciliature; PMT, posterior main tier; PR, plate processes; PS, posterior spine; PST, posterior secondary tier; W, plate windows. Scale bars=50 μm (A, D), 30 μm (E, F), 40 μm (G, H).

three plate processes, and triangle-shaped anterior end; usually two of them with anterior spines located at the same side (Figure 1A, B; Figure 2E). Anterior main plates approximately 40 μm long, with eight windows gradually enlarged from anterior to posterior, nine plate processes, and eight ciliary outlets (Figure 1B; Figure 2D). Posterior main plates approximately 30 μm long, with six windows, seven plate processes, and six ciliary outlets (Figure 1B; Figure 2C). Posterior secondary plates trapezoidal, about 12 μm long, with two elongated windows, three plate processes, and two ciliary outlets (Figure 1B). Caudal tier visible only in oblique or posterior polar view, about 5 μm long, usually with three small and sharp posterior spines (Figure 1A, B; Figure 2F). The fine structure of the armor plates is shown in Figure 1B; 2C-D: left margin generally smooth and slightly convex at the level of the bridge; sharp plate processes arranged alternately with windows on the right edge, connected with conspicuous bridges; windows "8" shaped with small radian sieve domes, separated in pairs by a midbar; generally, 2, 8, 6, 2 windows in the middle four tiers from the anterior to posterior (Figure 1A; Figure 2A).

Oral opening at anterior end of cell. Circumoral kinety circular, composed of dikinetids, interrupted at the site of three adoral organelles. Adoral organelles short and obliquely arranged. Organelle 1 composed of three pairs of kinetosomes, organelles 2 and 3 each composed of four pairs of kinetosomes (Figure 1C; Figure 2H). Internal and external oral basket in center of mouth, about 6 μm and 10 μm long, respectively, conspicuous in protargol-impregnated specimens (Figure 2G).

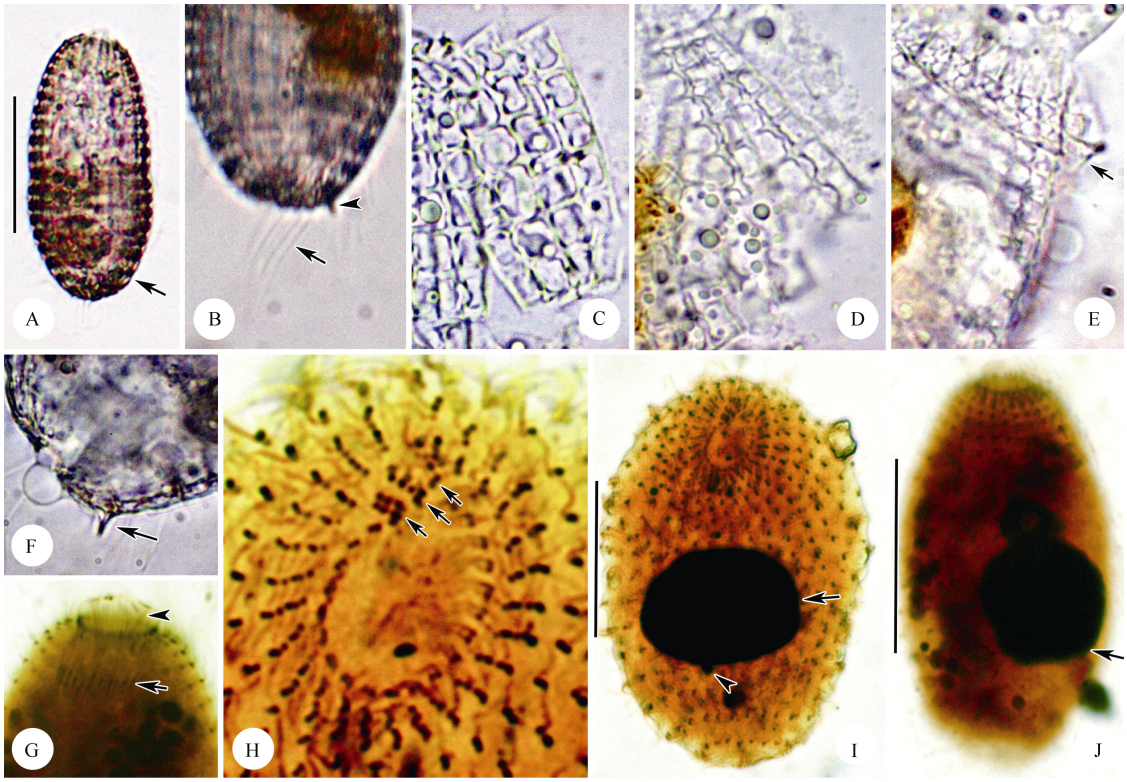

Figure 2 Microphotographs of living *Coleps amphacanthus* and after protargol impregnation

A: Lateral view of a typical individual, arrow indicates contractile vacuole. B: Lateral view of posterior section of the body, arrow marks caudal cilia, arrowhead denotes posterior spine. C: Posterior main plates. D: Anterior main plates. E: Lateral view of the anterior section of the cell, showing anterior spine (arrow). F: Lateral view of the posterior end of the body, arrow indicates posterior spine. G: Lateral view of anterior portion, showing internal (arrowhead) and external (arrow) oral basket. H: Detailed infraciliature of the apical end, arrows denote three adoral organelles. I: Infraciliature of a pressed specimen showing ciliary pattern, arrow denotes macronucleus, arrowhead indicates micronucleus. J: Lateral view of a specimen on a permanent slide, arrow denotes macronucleus. Scale bars=50 μm.

Somatic cilia about 15 μm long and regularly arranged, forming 18 transverse circles and 25 longitudinal rows on average (Figure 1D; Figure 2I, J). Anterior two transverse circles with dikinetids closely arranged and forming perioral ciliature (Figure 1C; Figure 2H). Five to ten significantly long caudal cilia (about 25 μm) (Figure 1A; Figure 2B).

***Levicoleps biwae jejuensis* Chen et al., 2016 (Table 1; Figure 3 and 4)**

The genus *Levicoleps* was established by Foissner et al. (2008) with *Levicoleps biwae* as the type species. Chen et al. (2016) reported a South Korean subspecies, *L. biwae jejuensis*, from

Jeju Island. These two subspecies are very similar in morphological characters and infraciliature, except the number of caudal cilia. A Chinese population of *L. biwae jejuensis* is described here to clarify intraspecific variations.

Improved diagnosis. Size in vivo about (70-110) μm×(30-60) μm, barrel shaped. On average 21-24 ciliary rows, each composed of about 15-18 monokinetids and two perioral dikinetids; 4-10 caudal cilia. Anterior and posterior main plates each with six and five windows, respectively; anterior and posterior secondary plates each with two windows, respectively.

Voucher slides. Two voucher slides with protargol-impregnated specimens were deposited in the Laboratory of Protozoology, Ocean University of China (registration numbers: LU-20150622-02-01, 02).

Morphological description of Chinese population. Size about (85-110) μm×(40-60) μm *in vivo*, barrel-shaped body with a length to width ratio of about 2: 1, usually slightly narrowed in the mid-body where the main armor plates abut. Body margin conspicuously wavy due to window domes (Figure 3A; Figure 4A). Anterior end transversely truncated and crown-like, posterior end moderately rounded to slightly pointed (Figure 3A; Figure 4A-C). Macronucleus globular to slightly ellipsoidal with a length: width ratio of about 1.2, positioned near the mid-body and close to the cell periphery. Micronucleus spherical, about 2 μm in diameter, attached to macronucleus (Figure 3E, G; Figure 4H, I). Contractile vacuole about 10 μm across, located near the subterminal of the body (Figure 3A; Figure 4B). Cytoplasm colorless, usually containing several food vacuoles (5-15 μm in diameter) and numerous droplets (1-5 μm across) (Figure 3A; Figure 4A-C).

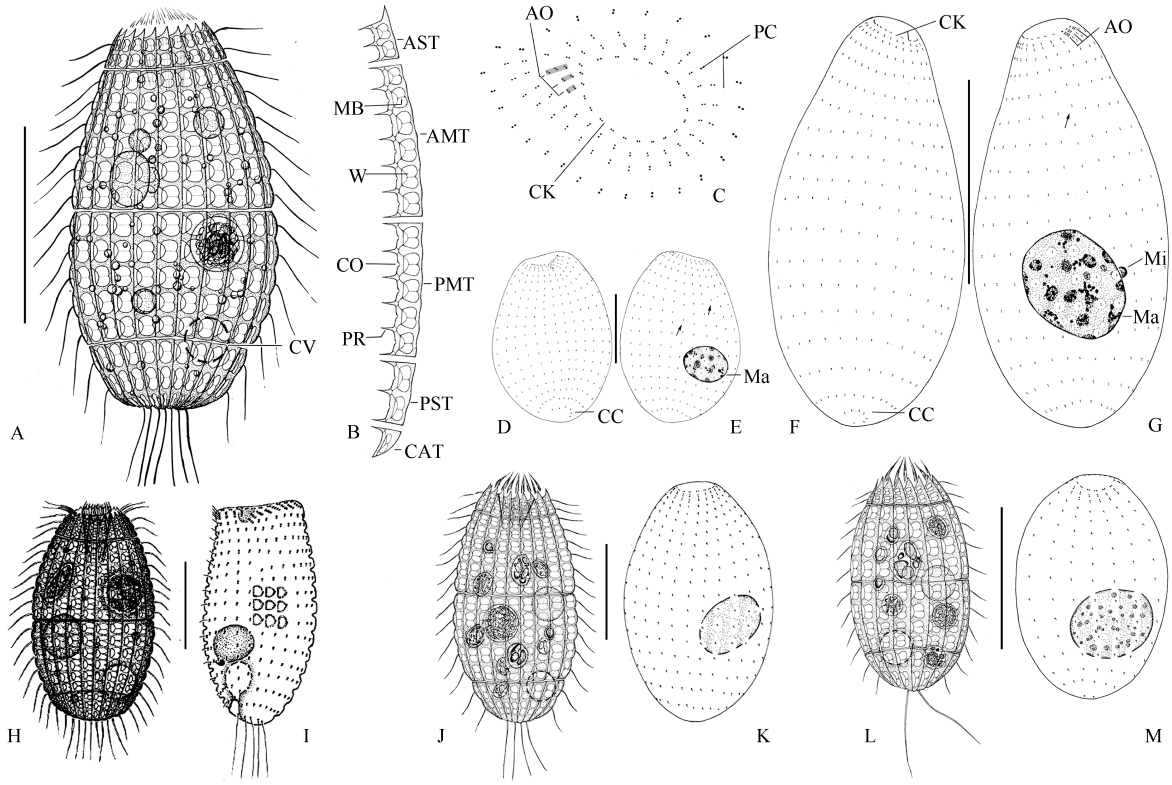

Figure 3 Morphology and infraciliature of the Chinese population (A-G) and South Korean population (J, K) of *Levicoleps biwae jejuensis*, *L. biwae biwae* (H, I), and *L. taehwae* (L, M)

A: Lateral view of a typical individual. B: A row of plates showing the fine structure of five tier plates. C: Ciliary pattern at apical end of the body. D, E: Lateral view of the infraciliature, arrows indicate irregular ciliary rows. F, G: Lateral view of the infraciliature, arrow indicates irregular ciliary row. H, I: Lateral view of living individual and infraciliature of *Levicoleps biwae biwae* (from Foissner et al., 2008). J, K: *Levicoleps biwae jejuensis* collected from Jeju Island (Chen et al., 2016). L, M: *Levicoleps taehwae* collected from Taehwae River, South Korea (Chen et al., 2016). Abbreviations: AO, adoral organelle; AMT, anterior main tier; AST, anterior secondary tier; CAT, caudal tier; CC, caudal cilia; CK, circumoral kinety; CO, ciliary outlet; CV, contractile vacuole; Ma, macronucleus; MB, midbar; Mi, micronucleus; PC, perioral ciliature; PMT, posterior main tier; PR, plate processes; PST, posterior secondary tier; W, plate windows. Scale bars=50 μm (A, D-G), 20 μm (H-M).

Armor plates *hirtus*-type and composed of six tiers: circumoral, anterior secondary, anterior main, posterior main, posterior secondary and caudal, with each tier composed of 21-24 plates (Figure 3A, B; Figure 4A-G). Circumoral tier hardly observed *in vivo*. Anterior secondary plates about 15 μm long, with triangle-shaped anterior end, two windows, three plate processes, and

two ciliary outlets (Figure 3B; Figure 4D). Anterior main plates about 35 μm long, with six windows gradually enlarged from the anterior to posterior, seven plate processes, and six ciliary outlets (Figure 3B; Figure 4F). Posterior main plates about 30 μm long, with five windows, six plate processes, and five ciliary outlets (Figure 3B; Figure 4G). Posterior secondary plates trapezoidal-shaped, about 12 μm long, with two elongated windows, three plate processes, and two ciliary outlets (Figure 3B; Figure 4E). Caudal tier about 4 μm long with a single window (Figure 3B). The fine structure of the armor plates is shown in Figure 3B and Figure 4D-G: left margin generally smooth and slightly waved at the level of the bridge, plate processes on the right edge arranged alternately with windows and connected with inconspicuous bridges; bi-window with conspicuous radian sieve domes. In general, 2, 6, 5, 2 windows in the middle four tiers from the anterior to posterior (Figure 3A, B; Figure 4A-G).

Oral opening occupying the central region of the anterior pole. Circumoral kinety circular and composed of dikinetids (Figure 3C; Figure 4J). Adoral organelles consisting of three parts, organelle 1 and 2 each composed of two pairs of kinetosomes, organelle 3 composed of three pairs of kinetosomes (Figure 3C; Figure 4J). Internal and external oral basket about 8 μm and 12 μm long, respectively, in protargol-impregnated specimens (Figure 4H).

Regularly arranged somatic cilia about 18 μm long, forming 15 transverse circles and 21-24 longitudinal rows (Figure 3D-G; Figure 4H, I). Anterior two close dikinetids of each longitudinal ciliary row forming perioral ciliature. Six to ten caudal cilia about 25 μm long *in vivo* (Figure 3A, D, F; Figure 4A, C, K).

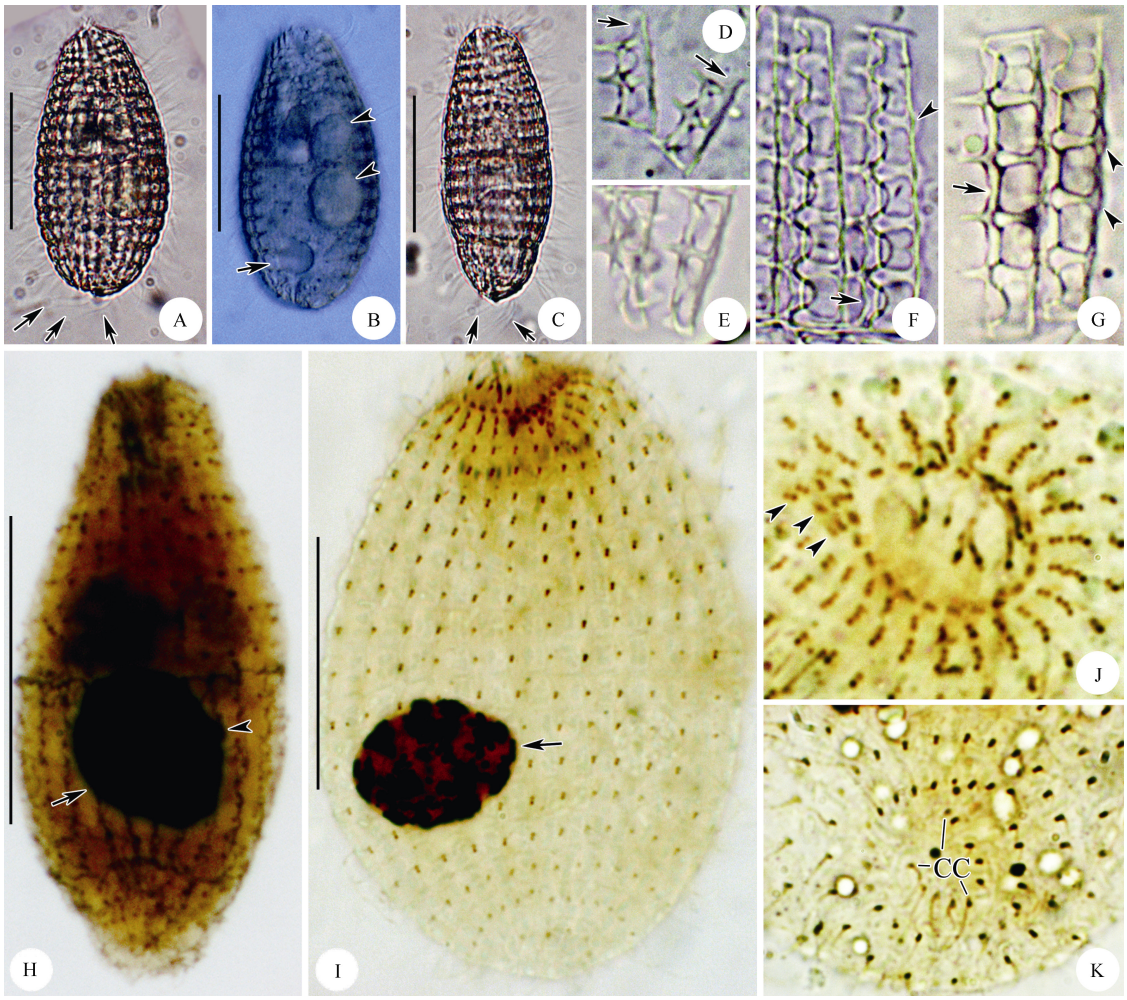

Figure 4 Microphotographs of the Chinese population of living *Levicoleps biwae jejuensis* and after protargol impregnation

A: Lateral view of a typical individual, arrows mark caudal cilia. B: Lateral view, showing contractile vacuoles (arrow) and food vacuoles (arrowheads). C: Lateral view of a slender individual, arrows denote caudal cilia. D: Anterior secondary plates, arrows indicate pointed anterior end. E: Posterior secondary plate showing elongated windows. F, G: Anterior main plates (F) and posterior main plates (G), arrows mark ciliary outlets and arrowheads mark bulge. H: Lateral view of infraciliature, arrow marks macronucleus and arrowhead denotes micronucleus. I: Lateral view of a pressed specimen after protargol-impregnation, showing ciliary pattern, arrow marks macronucleus. J: Apical view, showing ciliary pattern at apical end, arrowheads denote three adoral organelles. K: Bottom view, showing caudal cilia. Abbreviation: CC, caudal cilia. Scale bars=50 μm.

SSU rRNA gene sequences and phylogenetic analyses

The SSU rRNA gene sequences of *Coleps amphacanthus* and *Levicoleps biwae jejuensis* were deposited in the GenBank database with accession numbers KU525296 and KU525297, respectively. The length and GC content of the SSU rRNA gene sequence of *Coleps amphacanthus* were 1 718 bp and 44.18%, respectively, while those of *Levicoleps biwae jejuensis* (Ningbo population) were 1 719 bp and 44.27%, respectively.

Forty-seven species/populations were included in the present phylogenetic analyses, containing three oligohymenophorean species as out-groups and all species from classes Prostomatea and Plagiopylea available for SSU rRNA gene sequences. In the class Prostomatea, there were 34 species/populations representing 15 genera from seven families (Balanionidae, Colepidae, Holophryidae, Placidae, Plagiocampidae, Prorodontidae, and Urotrichidae). The topologies of the ML and BI trees were basically congruent and, therefore, a single topology was presented based on the ML tree with support values from both algorithms indicated on the branches (Figure 5). In the phylogenetic trees, Prostomatea was polyphyletic with all species grouped into three clades: Placidae formed one fully supported clade; Colepidae, Prorodontidae, and *Pelagothrix alveolata* (Holophryidae) formed the second clade (61% ML, 0.98 BI); Plagiocampidae, Urotrichidae, Balanionidae, and *Cryptocaryon irritans* (Holophryidae) formed the third clade

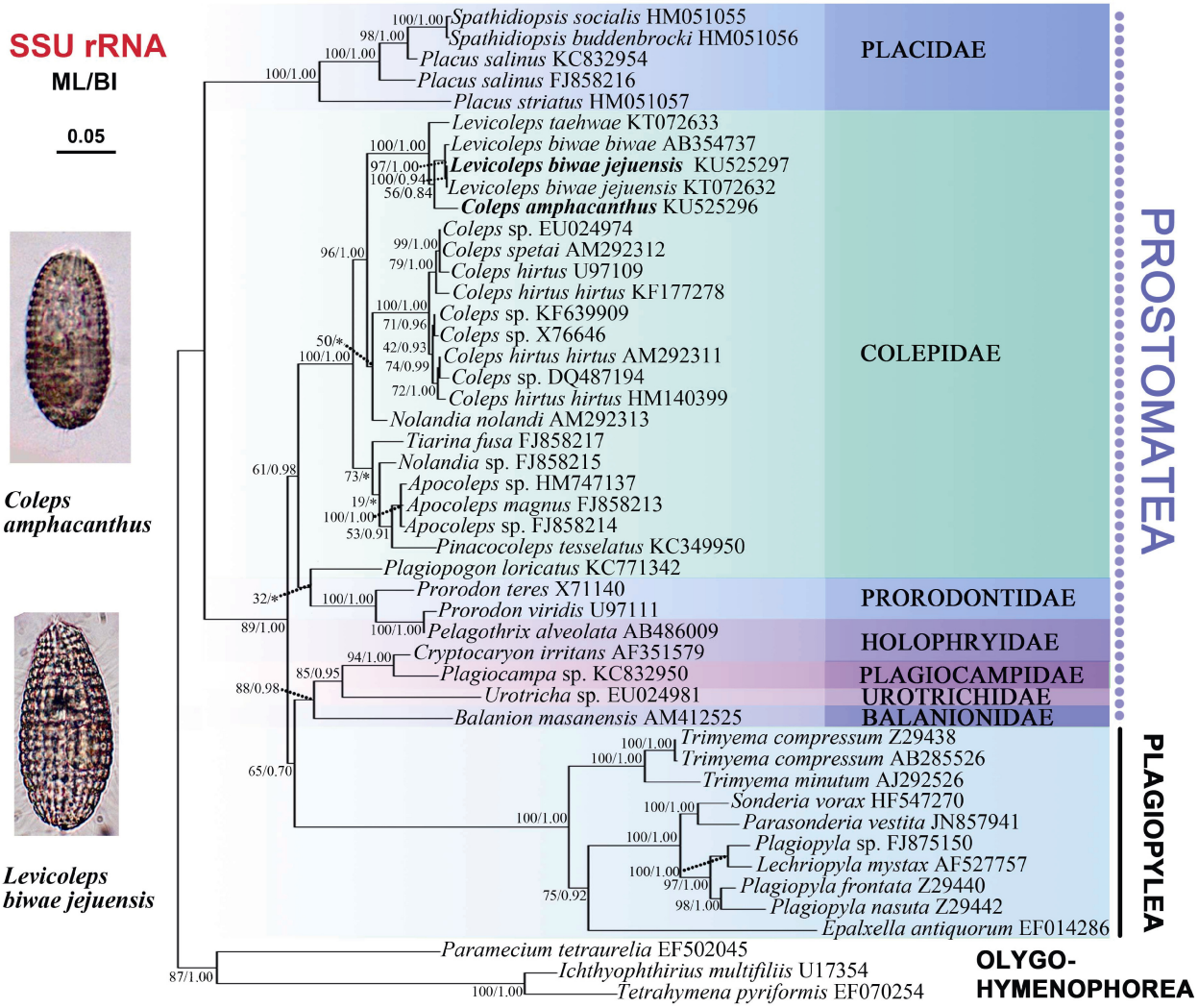

Figure 5 Maximum likelihood (ML) tree inferred from SSU rRNA gene sequences showing positions of *Coleps amphacanthus* and *Levicoleps biwae jejuensis*

Sequences newly obtained are in bold. Numbers near nodes represent non-parametric values of ML out of 1 000 replicates and Bayesian inference (BI) posterior probabilities. Disagreements between ML and BI are shown by asterisks. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

(88% ML, 0.98 BI), and is a sister to the class Plagiopylea (65% ML, 0.70 BI). Within the family Colepidae, both genera *Coleps* and *Levicoleps* were grouped together with high support (96% ML, 1.00 BI). All *Coleps* species were branched together, except for *Coleps amphacanthus*, which was grouped into the clade of the genus *Levicoleps*. All three *Levicoleps biwae* isolates formed a well-supported clade (97% ML, 1.00 BI), with the two isolates of *Levicoleps biwae jejuensis* grouped together.

Hypothesis testing

Neither the genus *Coleps* nor *Levicoleps* was a monophyletic group in the present phylogenetic tree, as *Coleps amphacanthus* was always nested within the *Levicoleps* cluster. However, the constrained topology that all species of *Levicoleps* formed a monophyletic clade was not rejected at the 5% significance level (P=0.127). The hypothesized monophyly of the genus *Coleps* was rejected (P=8e-008), which raises the question whether the current morphological classification of *Coleps* is appropriate. Additional molecular data from taxa with indistinct spines or several caudal cilia are needed to determine whether this genus should be further split (Table 2).

Table 2 Approximately unbiased test results

Topology constraints	Log-likelihood (-lnL)	AU value (P)
Unconstrained	14143.12163	0.967
Coleps	**14157.50543**	**8e-008**
Levicoleps	14251.18767	0.127

Significant differences (P<0.05) between the best maximum likelihood tree and constrained topologies are in bold.

DISCUSSION

Remarks on *Coleps amphacanthus* Ehrenberg, 1833

Coleps Nitzsch, 1827 is characterized by its spiny armor composed of six tiers with *hirtus*-type plates and three adoral organelles. Till now, only four species of *Coleps* have been investigated using silver staining methods: *Coleps amphacanthus* Ehrenberg, 1833, *C. elongatus* Ehrenberg, 1831, *C. hirtus hirtus* Nitzsch, 1827, *C. hirtus viridis* Ehrenberg, 1831, and *C. spetai* Foissner, 1984 (Foissner, 1984; Foissner & O'Donoghue, 1990; Foissner et al., 1994, 1999).

Coleps amphacanthus has been investigated several times based on living and fixed specimens (Ehrenberg, 1833; Huttenlauch, 1987; Hutternlauch & Bardele, 1987; Foissner & O'Donoghue, 1990; Kreutz & Foissner, 2006; Noland, 1925). However, standard taxonomic data were only provided by Huttenlauch (1987) and Foissner & O'Donoghue (1990). Except for smallish spines (*vs*. conspicuous spines in the original description and subsequent re-descriptions), the morphology of the present organism is identical with previous populations. The dissimilarity of the spines is possibly due to different environments (the definite conclusion can not be provided here, further studies on this character are necessary), and hence should be considered as a population-dependent character.

Coleps amphacanthus differs from most congeners by its large body size and non-single caudal cilium. Only one species, namely *C. elongatus*, has more than one caudal cilium and should be compared with *C. amphacanthus*. The latter can be easily distinguished from the former by the number of somatic kineties (24-26 in *C. amphacanthus vs*. 14-18 in *C. elongatus*), the number of windows in the anterior and posterior main plates (8, 6 in *C. amphacanthus vs*. 5, 4 in *C. elongatus*, respectively), and the number of caudal cilia (5-10 in *C. amphacanthus vs*. 2 in *C. elongatus*).

In the phylogenetic trees, *Coleps amphacanthus* was separated from the genus *Coleps*, and clustered in the genus *Levicoleps* (Figure 5). Foissner et al. (2008) suggested that the presence or absence of armor spines is one of four principal generic features in the family Colepidae, with *Levicoleps* differing from *Coleps* mainly by its smooth armor without any spines. As previously described, *Coleps amphacanthus* has several obvious anterior and posterior spines in the original and subsequent re-descriptions (Ehrenberg, 1833; Noland, 1925; Huttenlauch, 1986; Foissner & O'Donoghue, 1990). The Ningbo population of *C. amphacanthus* has two anterior and three posterior inconspicuous smallish spines, and therefore should be assigned to *Coleps* according to the possession of spines. However, phylogenetic analyses did not support this, indicating that the presence or absence of armor spines might not be a good generic feature to separate *Coleps* and *Levicoleps*. Unfortunately, no molecular information currently exists in regards to historic populations. Additional molecular data will help reveal the correct phylogenetic position of this organism.

Remarks on *Levicoleps biwae jejuensis* Chen et al., 2016

Levicoleps biwae was originally discovered in a 4-million-year-old ancient freshwater lake (Lake Biwa) in Japan (Foissner et al., 2008). Combined other biogeographic information, including the occurrence of *Planicoleps* only in Lake Tanganyika (Africa) (Dragesco & Dragesco-Kernéis, 1991), the genera *Baikalocoleps*, *Kotinia*, *Macrocoleps*, and *Tiarinella* seem to be restricted to Lake Baikal (Obolkina, 1995). Foissner et al. (2008) deduced that *Levicoleps* is a special genus from Lake Biwa. However, Chen et al. (2016) found a subspecies and other *Levicoleps* species, namely *Levicoleps biwae jejuensis* and *L. taehwae*, in South Korea. We also found a Chinese population of *L. biwae jejuensis* from a freshwater pond in Ningbo. In view of these isolations in South Korea and China, the genus *Levicoleps* does not appear to be endemic to Lake Biwa only, but is at least common to Asia.

Levicoleps is characterized by smooth armor composed of six tiers with *hirtus*-type plates, the absence of spines and three adoral organelles (Foissner et al., 2008). We identified our form by basic characters of armor plate, absence of spines, three adoral organelles, and several caudal cilia. Except for the number of caudal cilia and windows of the main armor plates, the organism we isolated corresponded perfectly with *L. biwae jejuensis*. At the present state of knowledge, these differences should be considered as population-dependent characters in *Levicoleps* species (Foissner et al., 2008). Moreover, this organism and *L. biwae jejuensis* (KT072632) were grouped together with strongly supported SSU rRNA trees (100% ML,

0.94 BI), and their SSU rRNA gene sequences differed by only one nucleotide. Based on morphological and molecular information, our isolate should be considered a Chinese population of *L. biwae jejuensis*. In addition, the SSU rRNA gene sequence of our isolate differed by seven nucleotides from that of *L. biwae biwae*.

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ZOOLOGICAL RESEARCH

Range extension of *Lepidocephalichthys alkaia* (Teleostei: Cobitidae) and notes on its sexual dimorphism

Marco ENDRUWEIT[1,2,*]

[1] *Kunming College of Life Sciences*, *University of Chinese Academy of Sciences*, *Kunming*, *Yunnan* 650204, *China*

[2] *State Key Laboratory of Genetic Resources and Evolution*, *Kunming Institute of Zoology*, *Chinese Academy of Sciences*, *Kunming*, *Yunnan* 650223, *China*

ABSTRACT

The natural distributional range of the cobitid loach *Lepidocephalichthys alkaia* is extended into Yunnan Province, China. The modified sexually dimorphic pectoral fin in males of *L. alkaia* is described.

Keywords: *Lepidocephalichthys alkaia*; Sexual dimorphism; Modified pectoral fin; Yunnan; China

INTRODUCTION

Spined loaches of the genus *Lepidocephalichthys* are a common component of lowland river fish assemblages in Indochina. They thrive abundantly in water bodies offering dense submerge vegetation and plenty woody debris. Species of the genus *Lepidocephalichthys* often represent the predominant benthic fish species in this type of habitat. Their abundance and neutral, non-muddy flavor of the flesh make them a part of subsistence fisheries in rural areas. Species of *Lepidocephalichthys* are diagnosed by having fused and hardened innermost pectoral-fin rays with a dorsal projection in males (Havird & Page, 2010; Šlechtová et al., 2008). So far, two species are known to occur in Yunnan, China; viz. *L. berdmorei* and *L. hasselti* (Chen, 2013; Havird & Page, 2010; Kuang, 1990). An ichthyologic survey in Dehong, Yunnan yielded a single specimen of *L. alkaia* from an agricultural market in Yingjiang town. Further specimens could be identified in the ichthyologic collection of the Kunming Institute of Zoology (KIZ), Kunming, China. In this contribution the sexually dimorphic, modified pectoral fin in males of *L. alkaia* is described.

MATERIALS AND METHODS

Meristics, morphometrics and related terminology follow explanations given in Kottelat (1990). Morphological abbreviations used: SL, standard length. Measurements are taken point to point with a caliper and recorded to the nearest 0.1 mm. Regional squamation pattern and morphological features were examined using a binocular Zeiss Stemi 2000-C at 20-50 times magnification.

Radiographs of the specimen were taken by a Kubtec Xpert 80 and used to count vertebrae and fin rays. Vertebral counts and associated terminology follow Roberts (1989); the terminal compound centrum supporting the hypural series is counted as one vertebra; the Weberian apparatus is counted as four vertebrae.

Specimen KIZ 2015000184 was preserved in the field using a 10% formalin solution and after five days transferred into 75% industrial ethanol for permanent storage. Institutional abbreviation used: KIZ, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China; ZRC, Zoological Reference Collection, Raffles Museum of Biodiversity Research, National University of Singapore, Singapore. Comparative data on *L. alkaia* are taken from Havird & Page (2010).

Lepidocephalichthys alkaia **Havird & Page, 2010**

Material: KIZ 2003004465-4475, 4477, 4478, 13 ex., 27.9-37.6 mm SL, Liangjiaoshui River, Irrawaddy basin, Longling County, Baoshan Prefecture, Yunnan, China; collected by X. Y. Chen, 16 September 2003. KIZ 2006010982-984, 986, 4 ex., 38.2-40.4 mm SL, same location; collected by X. Y. Chen & D. Neely, 27 April 2006. KIZ 2006010998-11004, 16 ex., 30.3-38.9 mm SL, Longchuanjiang River, Irrawaddy basin, Tengchong County, Baoshan Prefecture, Yunnan, China; collected by X. Y. Chen & D. Neely, 18 April 2006. KIZ 2015000184, 1 ex., 28.3 mm SL, Yingjiang town, Dayingjiang River, Irrawaddy basin, Yingjiang County, Dehong Prefecture, Yunnan, China; collected by M. Endruweit & T. Qin, 18 August 2014.

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[*]Corresponding author, E-mail: marco.endruweit@yahoo.de

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Figure 1 ***Lepidocephalichthys alkaia*****, KIZ 2015000184, 28.3 mm SL, male, lateral view**

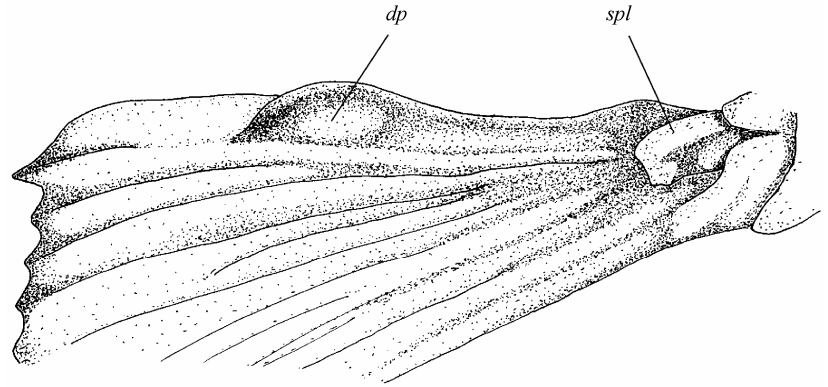

Figure 2 ***Lepidocephalichthys alkaia*****, KIZ 2003004465, 36.3 mm SL, schematized depiction of modified pectoral-fin rays, right side, dorsolateral view**

dp: dorsal projection; spl: suprapectoral lobe.

RESULTS

Material listed above was identified as *L. alkaia* based on the diagnosis given in its original description (Havird & Page, 2010): a midlateral dark stripe extends over the base of the caudal fin; caudal-fin shape truncate to round; top of the head scaleless; dorsal-fin origin posterior to pelvic-fin origin; and a moderate SL of up to 38 mm. Its chief morphometrics lie well within the ranges of *L. alkaia* with some slight deviations: predorsal length ranges from 51%-60% of SL in the type specimens *v.s.* 58-62 in the Chinese material, pre-pelvic length from 43%-54% of SL *v.s.* 50-55; and length of the pectoral fin from 13%-18% of SL *v.s.* 14-22. The number of unbranched rays in the dorsal and anal fins is given as two in the original description while it was counted to 2-3 on the radiographs. In addition, the radiographs showed a total vertebra number of 36 with 24-25 abdominal and 11-12 caudal vertebrae [*n*=10]. The largest type specimen is one of the three paratypes of lot ZRC 51544 measuring 38.2 mm SL. Specimen KIZ 2006010984 (40.4 mm SL; male) is slightly larger.

DISCUSSION

The herein reported occurrence of *L. alkaia* in Dehong and Baoshan prefectures raises the number of *Lepidocephalichthys* species in Yunnan to three. The occurrence of *L. alkaia* in Yunnan is expected since its type locality at Myitkyina in the Burmese Kachin State is adjacent and *Lepidocephalichthys* typically possesses a high dispersal rate with a wide distributional range provided that their habitat preferences are met. While the type series is exclusively described from the Irrawaddy basin Havird & Page (2010) list additional non-typic material from the Burmese Salween basin indicating a wide dispersal of *L. alkaia*.

A lamina circularis sensu Rendahl (1930, 1933) is a thin, osseous, saucer-like, horizontal projection of the second enlarged pectoral-fin ray. It is located proximal on the ray and is sexually dimorphic present exclusively in males of numerous cobitid species. The term ‘lamina circularis’ has been misused in recent ichthyologic literature (Das et al., 2012; Havird & Page, 2010) for the modified innermost pectoral-fin rays featuring a dorsal projection in males of *Lepidocephalichthys*. Yet a lamina circularis is absent in species of this genus.

The structure of the modified pectoral-fin rays in males of *L. alkaia* was not described nor depicted by Havird & Page (2010) since the original description based on an all female batch of 28 specimens. The two innermost pectoral-fin rays are fused and build a conspicuous, dorsally projected, hardened flange over approximately 2/3 of the rays’ length. The flange’s shape as depicted in Figure 2 is peculiar and diagnostic on species level. Adjacent to the flange there is a conspicuous, ovoid, fleshy

suprapectoral lobe adnate to the rays that is located proximally and may not be confused with a lamina circularis.

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